The role of tumor suppressor IKZF1 in leukemia development and therapy resistance

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About the cover:
Ikaros was the son of the inventor Daedalus, as handed down from ancient greek mythology. Daedalus produced technical miracles such as the maze of Crete and Pasiphae's Wooden Cow. Unarguable the most memorable invention of Daedalus were wings crafted out of wax and feathers, thereby granting a human the ability to fly. Ikaros, blinded by illusions of grandeur, dared to fly too close to the sun with his father's invention. When the sun finally melted his wings of wax, Ikaros plunged into the Greek Sea and tragically drowned.

The role of tumor suppressor IKZF1 in leukemia development and therapy resistance

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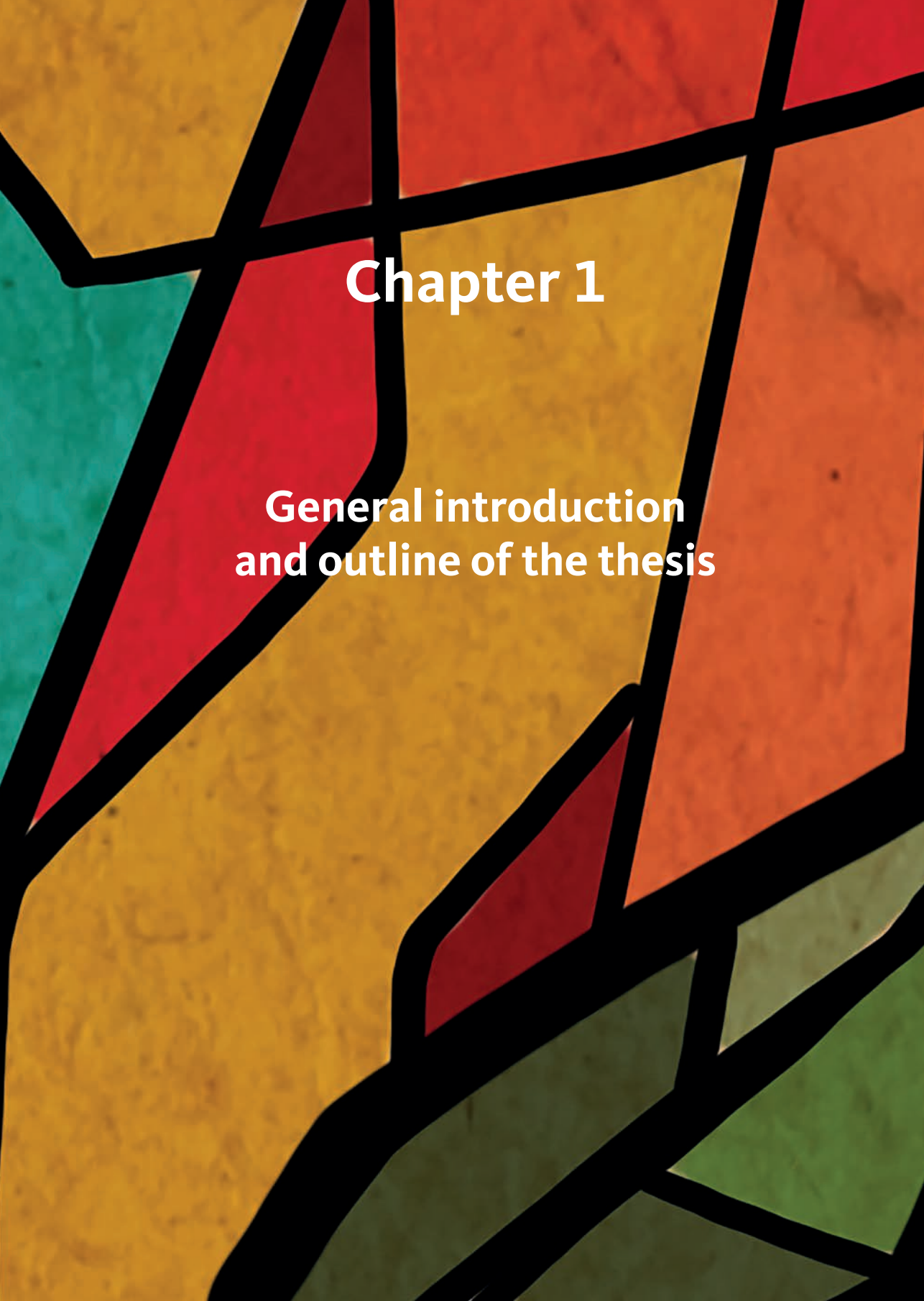
"Icarus,

Icarus, where are you? In what region shall I seek you?

Icarus!"

(Ovidius, Metamorphoses VIII, sentence 231-233, AD8)

Table of Contents



Chapter 1

General introduction and outline of the thesis

Overview

Genetic alterations leading to inactivation of the transcription factor IKZF1 (IKAROS) are present in about 15% off all patients with B-cell precursor leukemia (BCP-ALL). We and others have shown that genetic aberrations affecting IKZF1 represent a highly relevant clinical marker for poor outcome in BCP-ALL. However, how loss of IKZF1 function contributes to an unfavorable outcome remains poorly understood. In this thesis, we therefore investigated the link between loss of IKZF1 function and resistance towards commonly used chemotherapeutic agents in BCP-ALL, such as synthetic glucocorticoids and pyrimidine analogs. Next to this, additional genetic events may either enhance or negate the effects of IKZF1 deletions on outcome. Therefore, we examined which other co-occurring genetic events affect therapy response and outcome in *IKZF1*-deleted BCP-ALL.

1 | Hematopoiesis

1.1 | Blood cell formation and hierarchy

The formation of blood cells in the bone marrow is known as hematopoiesis, which is derived from the two ancient greek words αἷμα *haíma* "blood" and ποίησις poíēsis "to make". The major site of blood cell production changes during human development. In the developing embryo, the fetal liver is the main location for blood cell development. Prior to birth, the major site of blood cell development shifts to the bone marrow compartment [1]. During hematopoiesis, all blood cell lineages are derived from a small pool of self-renewing hematopoietic stem cells (HSCs) (Figure 1). This HSC pool is capable to reconstitute the entire blood system of an organism by expanding and differentiating to more restricted multipotent progenitors (MPPs), which branch into either common myeloid progenitor (CMP) or common lymphoid progenitor (CLP) cell populations [2]. These two branches further subdivide, in which CMPs differentiate into granulocyte-monocyte progenitors (GMPs) and megakaryocyte-eryhtroid progenitors (MEPs), thereby giving rise to erythrocytes, and platelets, whereas CLPs ultimately form the lymphoid part of the immune system, represented by NK cells, T cells and B cells [3].

1.2 | B-cell development

B-cells represent an important part of the adaptive immune system. Their main function is to provide the humoral component of the immune response against pathogens by producing antibodies, capable of recognizing and binding specific foreign antigens. Next to this, B-cells can also function as antigen presenting cells (APCs) [4] and produce cytokines such as lymphotoxin, interleukin 6 (IL-6), interferon-γ, tumor necrosis factor (TNF), IL-10 and IL-35 [5]. Originating from the bone marrow, immature B cells undergo their first maturation steps in the bone marrow niche, before they further migrate to

germinal centers in peripheral lymphoid organs to ultimately mature and take part in the immune defense [6]. During the differentiation process, proper development of B cells is strictly regulated at distinct developmental stages and checkpoints orchestrated by B cell transcription factors such as EBF1, PAX5, PU1, E2A and IKAROS gene family members [7-9] (Figure 2). These transcription factors control VDJ recombination and the subsequent formation of the B cell receptor (BCR) [10]. Only after a functional BCR is present on the surface of a B cell, it will leave the bone marrow and migrate to peripheral lymphoid organs.

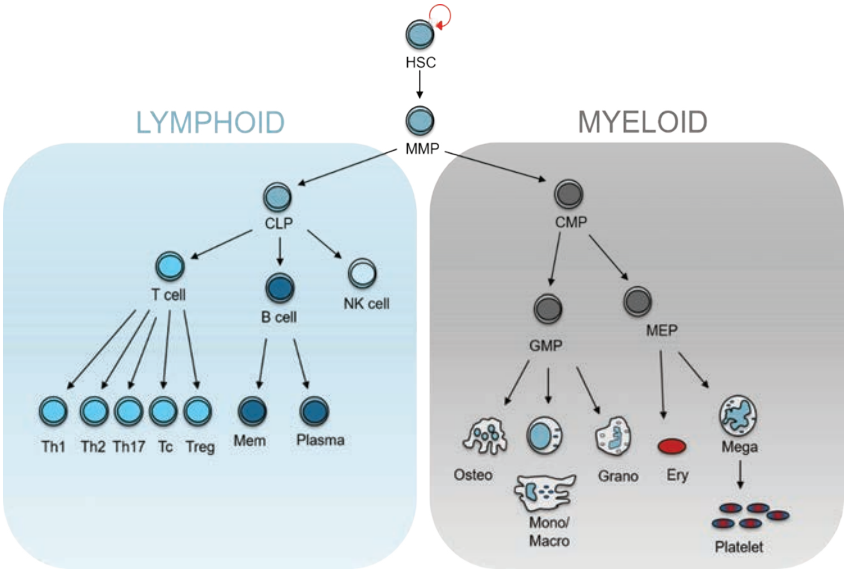


Figure 1 Schematic overview of haematopoiesis. Simplified model of blood cell lineage commitment from HSC differentiation to multipotent progenitors (MMP), followed by further branching into common myeloid precursors (CMP) and common lymphoid precursors (CLP). The CMP generate granulocyte/macrophage precursors (GMP) that yield granulocytes (Grano), monocyte/macrophages (Mono/macro) and osteoclasts (Osteo), as well as megakaryocyte/erythroid precursors (MEP) that yield megakaryocytes (Mega), platelets and erythroid cells (Ery). The CLP generate T cell precursors that produce three types of T helper cells (Th1,2&17), regulatory T cells (Tregs) and cytotoxic T cells (Tc), B cell precursors that produce memory and plasma cells, as well as Natural Killer (NK) cells. Adapted from [11]. Recent advances in deciphering the hematopoietic dynamics are more and more challenging this rigid developmental dogma and indicate more fluidity of the blood development [12]. To simplify this complex topic, we only refer to a simplified model of hematopoiesis in this figure.

The Ikaros family of zinc-finger proteins, whose names are inspired by Greek mythological characters, consist of IKZF1 (Ikaros), IKZF2 (Helios), IKZF3 (Aiolos), IKZF4 (Eos) and IKZF5 (Pegasus) [13-16]. The members of the Ikaros family play a crucial role during lymphoid differentiation and are highly conserved between mice and humans [17-19]. Ikaros family members contain several conserved functional domains including two C-terminal zinc-finger domains, required for homo- and/or heterodimerization with other Ikaros family

members and an N-terminal DNA binding domain [20]. *IKZF1* (Ikaros), which is located on chromosome 7p12.2, is the founding member of the Ikaros family, and a key determinant of early lymphoid lineage specification during hematopoiesis [21-23]. IKZF1 is required for the transition from HSCs to CLPs [14], but also at later stages of lymphoid (B cell) development [24]. Importantly, *IKZF1* plays a critical role in the pathogenesis of leukemia and deletions in the *IKZF1* gene are associated with adverse prognosis in BCP-ALL (See 2.3)

Further downstream, *E2A* and *EBF1* are essential for the generation of pro-B-cells. E2A promotes the development of LMPPs, CLPs, ETPs and controls all B-cell differentiation stages [25,26]. Simultaneously, *E2A* represses myeloid/erythroid lineage commitment and induces *EBF1* expression [27,28]. *EBF1* functions downstream of *E2A* during B-cell maturation and is essential for the expression of B-lineage committing genes, such as *FOXO1* and *PAX5* [26]. Later, *EBF1* maintains the identity of follicular and germinal center B-cells [28-30]. At later stages, *PAX5* and *PU.1* are necessary to maintain proper B cell identity by repressing non-B-cell lineage genes, while activating B-cell lineage genes [31-35].

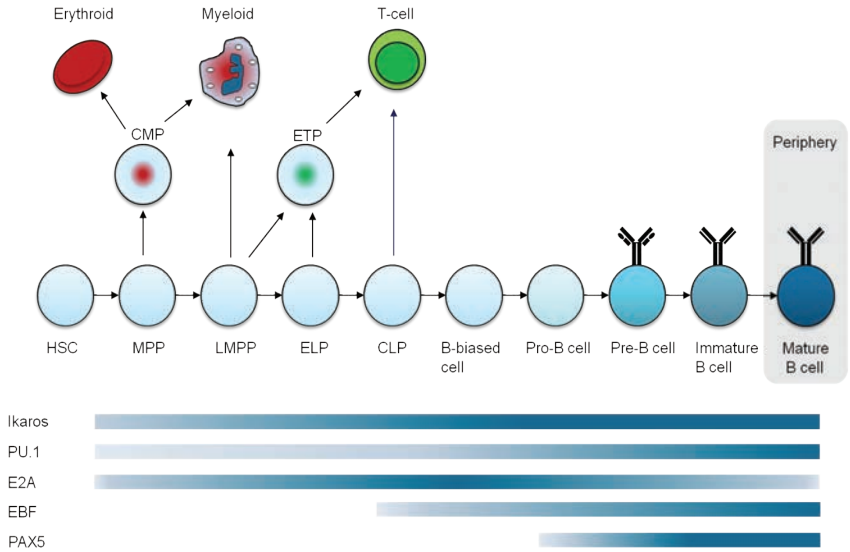


Figure 2 Transcription factor expression during B lymphopoiesis. The progression of cells from hematopoietic stem cells through the different stages of B lymphopoiesis is shown in this simplified model. Shaded bars represent the levels of gene expression of transcription factors that are important in B cell development during the course of differentiation. HSCs (hematopoietic stem cells), MPPs (multipotent progenitors), LMPPs (lymphoid-primed MPPs), ELPs (early lymphoid progenitors), CLPs (common lymphoid progenitors), ETPs (early T lineage progenitors). Darker shading indicates increased gene expression. Important branch points during B lymphopoiesis are shown with arrows indicating alternative developmental pathways. Adapted from [36].

2 | Leukemogenesis

2.1 | Introduction

Over the course of a day, human hematopoiesis is responsible for the proper development of approximately 10^{12} blood cells [37]. A strict regulation of differentiation and proliferation of all these blood cells is crucial to ensure proper development of the hematopoietic system. This balanced process is perturbed in leukemia, which is defined by the unsuppressed production of large numbers of immature white blood cells that fail to differentiate into mature functional lymphoid cells [38]. This malignant expansion of immature B-cells, so called blasts, in the bone marrow leads to a subsequent infiltration of the peripheral blood. As a consequence of this uncontrolled expansion, normal blood cell development is suppressed resulting in a defective immune system, anemia and/or thrombocythemia.

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children according to the World Health Organization classification, with an estimated 4930 new cases in 2016 in the US [39]. In the Netherlands, on average 130 children are diagnosed with ALL every year [40]. Approximately 15-20% of the patients succumb to the disease mainly due to relapse and associated therapy resistance [38,41]. Leukemias can be classified based on their lineage of origin, lymphoid or myeloid [41]. Amongst the lymphoid malignancies, approximately 85% of all patients develop B-cell leukemia, whereas the remaining 15% are T-cell lineage leukemias [42].

2.2 | Prognostic factors in pediatric leukemia

Several prognostic factors are related to the overall chance of survival of a patient with lymphoblastic leukemia treated with current, multi-agent chemotherapy: Immunophenotyping of the disease (B or T cell leukemia) [43], age at initial diagnosis [44] and response on treatment (e.g., defined by day 8 prednisone response [45] and minimal residual disease (MRD) status [46-48] after Induction and/or consolidation treatment) are important parameters for risk stratification of ALL patients [49]. However, increased insight into the genetic make-up of leukemia has also contributed to improved prediction of disease outcome during the last decades.

Cancer is as a disease characterized by an accumulation of genetic aberrations. In comparison to other cancers, pediatric leukemias display a low number of somatic mutations [50]. In addition to specific karyotypes, both gene expression signatures and certain genetic alterations play a role in ALL prognosis. Especially in the last decade, complementary genome-wide screening approaches made it possible to identify genetic drivers contributing to leukemia development. [51,52]. Many of these aberrations affect crucial cellular processes, such as lymphoid development, cell cycle progression, epigenetic changes and cellular metabolism [51-53].

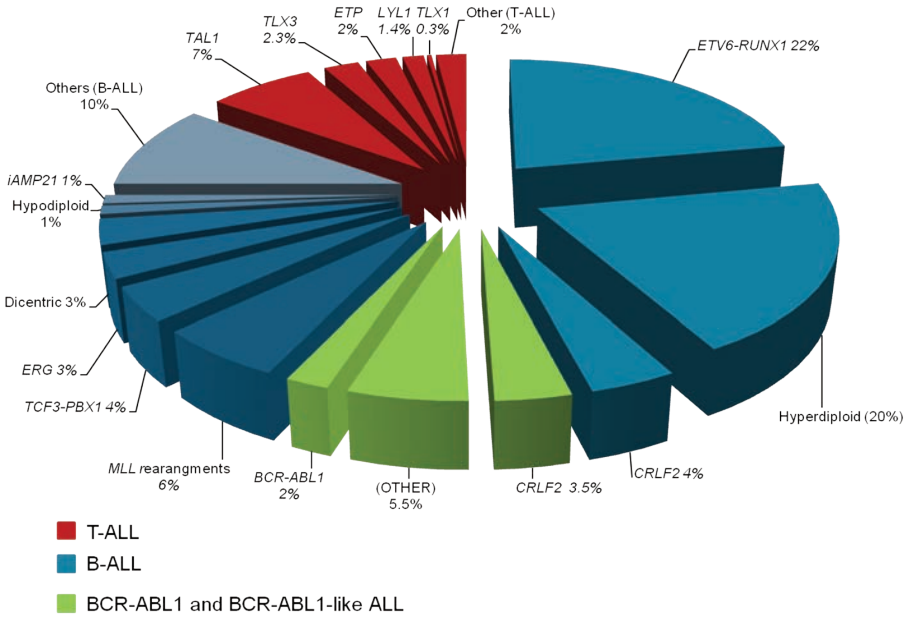


Figure 3 Frequency of cytogenetic subtypes of pediatric ALL. T-cell acute lymphoblastic leukemia (T-ALL) is indicated in red, B cell acute lymphoblastic leukemia (B-ALL) in blue and both BCR-ABL1 and BCR-ABL1- like ALLs are indicated in green. Adapted from [54].

In BCP-ALL, B-lineage transcription factors (See 1.2) are frequently deregulated and affected by either chromosomal translocations, gene deletions or point mutations [54].

Several genetic rearrangements, each with a different prognostic impact, have been identified in BCP-ALL (Figure 3). For example, chromosomal translocations involving the ETV6 and the *RUNX1*-gene found in almost 22% of all BCP-ALL cases [55-57] (*TEL-AML1* fusions) correlate with good overall survival of leukemia patients [58], whereas *BCR-ABL1* and *MLL*-rearrangements predict poor treatment outcome [59]. The fusion of *BCR* to the *ABL1* locus (t9;22)(q34;11) leads to a constitutive active ABL1 tyrosine kinase, thereby strongly enhancing proliferation of leukemic cells [60]. Therefore, *BCR-ABL1* is considered to be an initiating driver lesion in leukemia. Next to translocations, also alterations of chromosome numbers are related with disease outcome: Hyperdiploidy is a genetic event observed in approximately 30% of all pediatric BCP-ALL patients and is defined by the presence of more than 50 chromosomes per leukemic cell [61]. Microdeletions in *ETV6*, *CDKN2A* and *PAX5*, as well as point mutations of *FLT3*, *NRAS*, *KRAS* and *PTPN11* have been identified in this subgroup [61]. Hyperdiploidy is correlated with a favorable prognosis, whereas hypodiploids with fewer than 45 chromosomes predict a poor outcome [59].

Recently, a novel genetic group associated with poor survival has been identified in BCP-ALL [62,63]. The so-called *BCR-ABL1*-like subtype represent leukemias with a genetic expression profile similar to BCR-ABL1 positives cases, but without a detectable *BCR-ABL1* rearrangement. Identified simultaneously by independent groups, these leukemias are characterized by activation of various kinase and cytokine pathways combined with a high frequency of aberrations in B-cell differentiation genes such as *EBF1*, *PAX5* and *IKZF1* [62,63].

2.3 | IKZF1 in leukemia

When studying the biological function of affected genes in BCP-ALL, it becomes apparent that many deletions in BCP-ALL can be found in genes encoding lymphoid transcription factors [51,52]. The important role of IKAROS family members as key determinants of lymphoid development evidently implies their involvement in the development and onset of hematopoietic malignancies. Genomic profiling of ALL patients indeed revealed that deletions of Ikaros transcription factor family members are frequently detectable alterations in leukemia [51,52]. Surprisingly, despite the importance of all IKAROS family members in lymphoid development and differentiation, only *IKZF1*, *IKZF2* and *IKZF3* deletions have been shown to be present in different types of leukemia up to now. Loss of *IKZF2* has been found in adult T-cell ALL and low hypodiploid BCP-ALL [64,65]. However, the role of IKZF2 in pediatric leukemia is barely studied. *IKZF3* gene deletions or point mutations have been detected in about 2% of all BCP-ALL cases, especially in near-haploid ALL [52], which also harbor alterations targeting receptor tyrosine kinase- (RTK-) and Ras signaling [65]. Despite their role in T-cell development [19], both *IKZF4* and *IKZF5* have not been reported to contribute to leukemogenesis or to have any prognostic value in a clinical setting.

Deletions and mutations affecting *IKZF1* have been found in about 10–15% of BCP-ALL and 4% of T-ALL cases [51,52] and *IKZF1* aberrations are highly enriched in BCR-ABL1 positive leukemia [66] and BCR-ABL1-like cases [62,63]. Importantly, a number of studies from our laboratory and others have shown that deletions and inactivating mutations targeting the lymphoid transcription factor gene IKZF1 represents a strong risk factor for poor outcome and the development of relapse in BCP-ALL [63,67,68]. The majority of *IKZF1* deletions impact the whole gene resulting in haplodeficiency, or delete the DNA-binding domain residing in exon 4-6 or 4-7, leading to expression of dominant-negative isoforms of *IKZF1*. This so called IK6-isoform of *IKZF1* impairs the function of IKZF proteins in a dominant-negative manner and results in a loss of the tumor suppressor function of IKZF1 [69].

Moreover, *IKZF1* deletions are associated with several high-risk features, for example high white blood cell count (WBC), age and persistent MRD [63,70,71]. Aberrations of *IKZF1* can be detected in the majority of cytogenetic subgroups in BCP-ALL: *IKZF1* deletions (i) are present with a low frequency of *ETV6-RUNX1* t(12;21)(p13;q22) positive cases (3-6%) [72,73] (ii) are overrepresented in *BCR-ABL1* positive cases (75%) [66] and (iii) can be found

highly enriched in the so-called "B-others" subgroup (39%) [62]. Notably, *IKZF1* deletions in both B-others as well as BCR-ABL-positive cases are highly associated with poor outcome [74,75]. In addition, *IKZF1* is reported to be frequently mutated in high hyperdiploid BCP-ALLs (20%) [61,74]. In summary, *IKZF1* gene deletions represent an independent prognostic factor that predicts inferior treatment outcome in different cohorts of childhood and adult BCP-ALL [63,68,76-78].

2.4 | The importance of genetic context in IKZF1-deleted leukemia

Although *IKZF1* aberrations contribute to leukemia onset, it is evident that loss of *IKZF1* alone is not sufficient to induce leukemogenesis [79,80]. This leads to the assumption that *IKZF1* aberrations are secondary events in leukemia formation, acting in combination with initiating events such as *BCR-ABL1* translocations [81] or a *BCR-ABL1*-like genetic context such as activated JAK-STAT signaling [82]. Co-occurrence of deregulated *CRLF2* and *JAK2* mutations can also be detected in *IKZF1*-deleted BCP-ALL cases and may also increase the risk of relapse [74,83]. However, not all *IKZF1*-deleted leukemias relapse, arguing that other cooperative genetic events can alter the survival outcome of *IKZF1*-deleted ALL. For example, deletions of the ERG gene have been reported to counteract the rather poor prognosis of *IKZF1*-deleted leukemia [84]. Besides *IKZF1*, many other recurrent genetic aberrations have been observed in BCP-ALL, which include deletions affecting *BTG1*, *CDKN2A/B*, *EBF1*, *ETV6*, *PAX5*, *RAG1*, *RB1* and *TCF3* [51,52]. From this, it seems that alterations in B cell development genes, leading to a block in B cell differentiation, are almost obligatory steps in the development of ALL (Also see 2.2).

Amongst these B cell developmental genes, a frequently affected gene in BCP-ALL is the transcriptional co-regulator B cell translocation gene 1 (BTG1), which occurs in about 9% of all pediatric BCP/ALL patients [51,52]. BTG1 was originally identified as a translocation partner of the *c-MYC* gene in chronic lymphocytic leukemia and is part of a family of antiproliferative genes, including *BTG2*, *BTG3*, *TOB* and *TOB2* [85]. *BTG1* on its own has no proven enzymatic activity, but several studies suggest that BTG1 functions as an adaptor molecule or as a cofactor involved in transcriptional regulation. It was shown to interact with homeobox transcription factor 9 [86], the transcriptional regulator CAF1 [87] and with the arginine methyltransferase PRMT1 [88,89]. Next to this, we have established *BTG1* as crucial regulator of glucocorticoid receptor autoinduction in acute lymphoblastic leukemia [90].

3 | Therapy resistance in BCP-ALL

3.1 | Treatment of children with leukemia

The administration of chemotherapy in the treatment of children with acute lymphoblastic leukemia consists of induction of remission (induction phase), intensification (consolidation) and maintenance phase. The goal of remission induction is to effectively eliminate the bulk of leukemic cells, driving the disease into remission. However, despite the fact that initial induction of remission indicates a good prognosis of the overall disease outcome, the initial achievement of remission does not mean the patient is cured [91]. The persistence of residual leukemic cells (often called ´Minimal´ residual disease (MRD)) is one of the main causes of relapse in ALL and quantification of MRD is an important indicator for a successful early response to therapy [92-94].

The current ALL-11 protocol of the Dutch childhood oncology group consists of a combination of different classes of (chemo-)therapeutic agents to effectively eradicate all residual leukemic cells [95]. Amongst them are synthetic glucocorticoids (prednisone, dexamethasone) [96], DNA damaging agents (for example daunorubicin) [96], cell cycle arrest therapies (vincristine) and amino acid depleting drugs (asparaginase) [97]. Furthermore, patients can receive methotrexate, AraC and 6-mercaptopurine (6-MP) or 6-thioguanine (6-TG) [98,99]. The average length of therapy for the ALL-11 medium risk group is 2 to 3 years, in which children with *IKZF1*-deleted leukemia receive one additional year of 6-MP and methotrexate due to a higher risk of relapse [95]. It is important to note that the aforementioned improved survival of children with ALL is almost exclusively due to better risk stratification, supportive care and strong improvements of both the dosage and the scheduling of chemotherapies rather than the development of novel drugs.

3.2 | Mechanisms of cellular drug resistance

Although most patients with ALL initially respond to therapy by achieving complete remission (CR), responses are not always lasting [100]. Even with the current standard of treatment for ALL, close to 15% of young leukemia patients ultimately succumb to the disease [101]. Despite the fact that chemotherapy effectively reduces the tumor burden, in case of relapse, cancer cells that are no longer sensitive to the therapy are able to expand [102]. As a consequence, relapsed leukemic blasts are often more resistant to therapy in comparison to leukemic blasts at diagnosis [103], making the treatment of relapsed ALL challenging [104].

The genetic composition of leukemia can change from diagnosis to relapse and the contribution of minor genetic subpopulations to chemotherapy resistance and thereby relapse formation is still highly discussed in ALL [105]. Next to this, small undetectable remaining populations of leukemic cells can avoid therapeutic killing during initial

treatment by adopting a dormant, and thereby non-active state [106]. These dormant, probably pre-leukemic stem cells were found to cause recurrence of relapse, sometimes even years after remission [106].

Several mechanisms for chemotherapy resistance in leukemia have been proposed and can broadly be divided into extrinsic and intrinsic mechanisms of resistance: Intrinsic resistance drug mechanisms involve specific properties of the leukemic cell, which are usually dictated by the genetic context of the leukemia [107]. For instance, activation and efflux of a chemotherapeutic agent, inhibition of apoptotic pathways, activation of DNA repair mechanisms and proliferation pathways as well as epigenetics are all contributing to intrinsic drug resistance [108]. In addition, extrinsic mechanisms contributing to drug resistance can be provided by the cellular microenvironment of the bone marrow, the so-called leukemic niche, in which interactions with stromal cells can enhance survival of leukemic cells during therapy [109]. Moreover, leukemic cells invading the central nervous system (CNS) can similarly escape therapy induced apoptosis by interaction with the CNS microenvironment [110,111] and because of poor penetration of many chemotherapeutic agents into the CNS [112]. All these factors can act either independently or in combination and can be mediated by a broad variety of signaling pathways.

During my thesis work, we focussed on **glucocorticoids** and **pyrimidine analogs**, which is why I will focus on these two classes of chemotherapeutic agents in the following segments.

3.3.1 | Glucocorticoids as cornerstone drugs in ALL treatment

Glucocorticoids (GCs) were among the first drugs used in the treatment of childhood acute lymphoblastic leukemia and have been the cornerstone of ALL treatment for more than 60 years. In 1948, synthetic versions of steroid hormones isolated from animal adrenal glands where shown for the first time to be effective in treating patients with rheumatoid arthritis [113]. Only two years later, the discovery of adrenal cortex hormones and their biological effects by Philip S.Hench, Edward Kendall and Tadeus Reichstein was awarded with the Nobel Prize in Physiology or Medicine. Since then, GCs have been introduced for the treatment of inflammation, autoimmune reactions and hematological cancers. Glucocorticoids are stress signaling hormones that regulate a broad variety of physiological processes [114]. The physiological and pharmacological effects of glucocorticoids are mediated by the glucocorticoid receptor (GR), also termed NR3C1, a member of the nuclear receptor super family of ligand-dependend transcription factors [115]. In its unoccupied and therefore inactive state, the GR resides in the cellular cytoplasm as part of a large multi-protein complex including chaperone proteins such as the head/shock proteins HSP90 and HSP70, SRC kinases, p23 and immunophilins [116,117] (Figure 4).

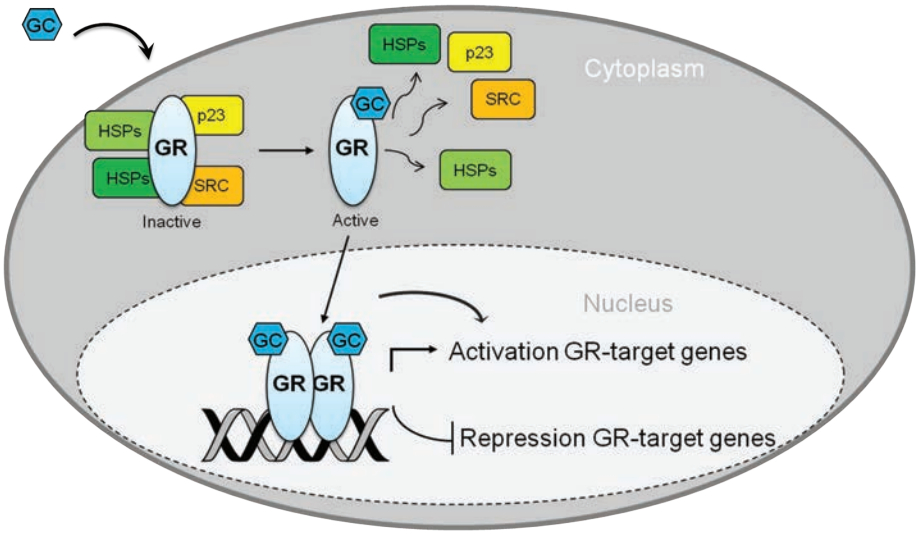


Figure 4 Simplified scheme of classical Glucorticoid Receptor (GR) activation. The GR remains in an inactive confirmation as a part of a large protein complex together with Head Shock proteins, p2, SRC kinases and others. After binding of GCs to the GR, the GR undergoes a conformational change and is able to translocate to the Nucleus, where it binds to DNA and either activates or inhibits GR-target genes.

Whereas the GR remains functionally silenced as long as it is bound in this complex, it holds a high affinity for GCs (Figure 4). Upon binding of GCs to its C-terminal ligand binding domain, the GR undergoes a conformational change, which allows translocation into the nucleus and bind other transcriptional regulators, such as activating protein (AP1) or nuclear factor kB (NFkB), thereby inhibiting their activity [118,119]. Alternatively, the GR is recruited to glucocorticoids response elements (GRES) in the nucleus [120], where it either activates or inhibits transcription, thereby regulating several downstream pathways involved in proliferation, survival pathways, NFkB signaling and glucose metabolism. In addition, several posttranslational modifications can affect GR signaling: The most studied GR modification is phosphorylation [121-123]. Various kinases such as Casein Kinase II, GSK3, AKT and MAPKs were shown to modulate GR signaling by phosphorylation of specific serine residues [124-126]. This leads to changes in the transcriptional activity of the GR, in which the phosphorylation of different serine residues can either drastically enhance or decrease the expression of GC-responsive target genes [127,128]. Next to phosphorylation, also sumoylation [129] and ubiquination [130,131] have been reported to affect transactivation and subnuclear trafficking of the GR.

3.3.2 | Resistance mechanisms against glucocorticoids

Studies performed by the Berlin-Frankfurt-Münster (BFM) group showed that the initial response to prednisone after seven days of single-agent treatment was strongly predictive of the overall survival outcome of patients [132]. Moreover, other studies indicated that ex vivo GC treatment of leukemic blasts was also effective in predicting disease outcome [103,133,134]. Therefore, both *in vitro* as well as *in vivo* resistance to glucocorticoids can be considered an adverse prognostic factor in ALL [45,134,135] and the resistance to synthetic GCs remains a substantial problem in the treatment of ALL patients.

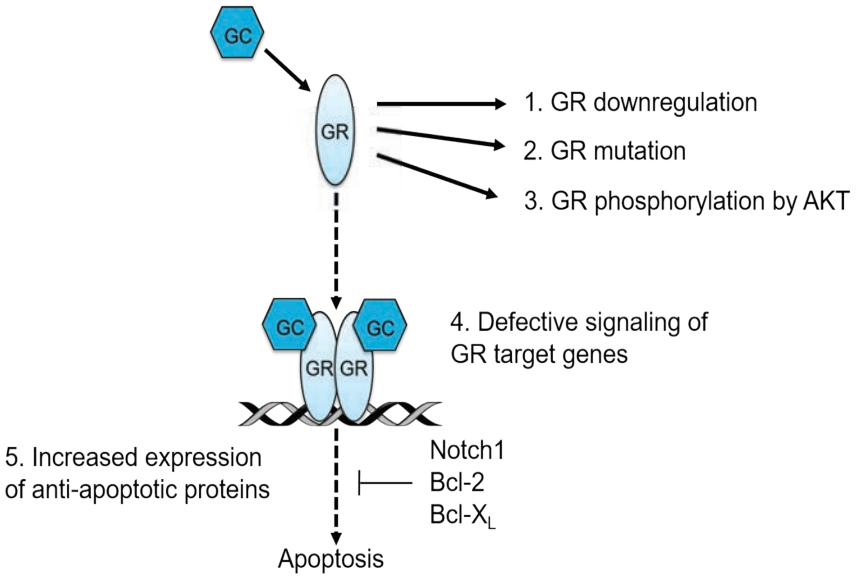


Figure 5 Mechanisms contributing to GC resistance in BCP-ALL

A number of studies have revealed potential mechanisms of GC resistance in ALL. For instance, it was shown that low expression levels and mutations of the *NR3C1* gene [136], epigenetic silencing of the pro-apoptotic gene *BIM* [137], downregulation of the anti-apoptotic BCL2, and increased activity of the Ser/Thr kinase AKT [138] can contribute to GC resistance (Figure 5).

Other studies have identified novel regulators of GR-dependent gene transcription that may impact GC therapy responses, including noncoding RNA GAS5 [139] GMEB1 and GMEB2 [140], BTG1 [90], histone methyltransferase G9a/KMT1C [141], TBL1XR1 [142] and metastasis-related methyltransferase 1 (MERM1)/WBSCR22 [143]. Finally, not all GR isoforms have the ability to induce apoptosis, suggesting that mechanisms that regulate isoform selection may also play a role [144]. Recent studies also indicate that PAX5 and IKZF1 can function as metabolic gatekeepers of a leukemic cell, thereby potentially changing therapy responses of a cancer cell [145].

3.4.1 | Pyrimidine analogs in the treatment of children with leukemia

Nucleoside analogs are commonly used in the treatment of leukemia, as the incorporation of these so-called antimetabolites into the DNA during DNA repair and replication induces apoptosis of cells. Amongst the different analogs, the cytidine analog Cytarabine (cytosine arabinoside (AraC)) is reported to be effective in the treatment of acute myeloid leukemia, both in adults and pediatric patients, already since the 1960s [146]. Notably, AraC is also implemented in ALL treatment protocols next to methrotrexate and thiopurines such as 6-MP or 6-TG [95]. After its transmembrane transport into the cytoplasm, AraC transits from AraC monophosphate (AraCMP) to AraC diphosphate (AraCDP) before it finally forms AraC triphosphate (AraCTP). This reaction is depending on the sequential phosphorylation by several kinases, amongst them deoxycytidine kinase (dCK). In contrast, the formation of AraCTP is opposed by the dephosphorylation of AraCMP into AraC by pyrimidine nucleotidase I (PN-I), the deamination of AraC into its inactive form, Ara-uridine (AraU), by cytidine deaminase (CDA) and the deamination of AraCMP into AraUMP by deoxycytidylate deaminase (dCMPD). In addition, AraCTP competes with deoxycytidine triphosphate (dCTP) for incorporation into DNA [147] (Figure 6).

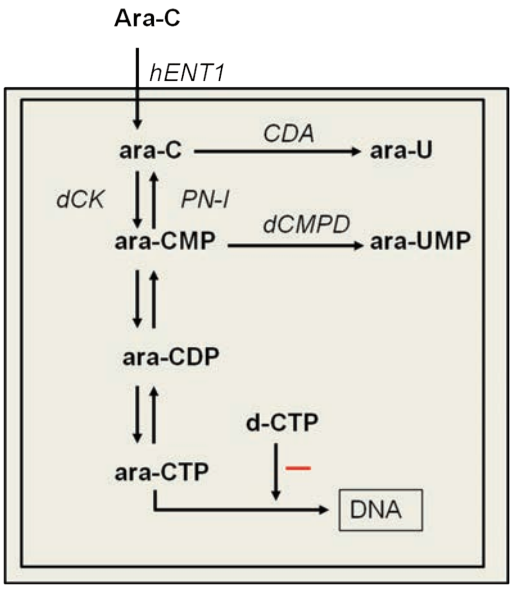


Figure 6 Symplified model of the cellular metabolism of AraC. Abbreviations: Cytosine arabinoside (AraC), human equilibrative nucleoside transporter, (hENT1) AraC monophosphate (AraCMP) , AraC diphosphate (AraCDP), AraC triphosphate (AraCTP). Cytidine deaminase (CDA), deoxycytidine kinase (dCK), pyrimidine nucleotidase I (PN-I deoxycytidylate deaminase (dCMPD), deoxycytidine triphosphate (dCTP). Adapted from [148].

3.4.2 | Resistance mechanisms against cytarabine

Despite intensive chemotherapy, 20% of pediatric patients with ALL do not achieve a complete remission or relapse after intensified chemotherapy [149]. AraC is an effective drug in the chemotherapeutic treatment of patients with acute myeloid leukemia [150,151] and acute lymphoblastic leukemia [152,153]. Still, the resistance to AraC therapy remains a substantial issue in the treatment of patients with leukemia [147].

Several mechanisms of AraC therapy resistance have been described in leukemia, in which all mechanisms decrease the availability of active AraCTP in the cell (Figure 7). First, reduced activity of the Human equilibrative nucleoside transporter (hENT1) has been linked to low cytoplasmic concentration of AraC [148]. hENT1 functions as transporter for several nucleoside analogs, such as AraC, gemcitabine, fludarabine and cladribine and transports up to 80% of total AraC into leukemic cells [154,155]. Also reduced levels of deoxycytidine kinase (DCK) render cells resistant towards AraC, as the initial phosphorylation step from AraC to AraCMP is decreased [156]. Seemingly, lower DCK levels are more relevant for AML, as several studies have shown that DCK levels in ALL patients did not correlate with AraC sensitivity [154,155]. On the other hand, increased levels of enzymes involved in AraC metabolism can be involved in nucleoside analogue resistance: It has been shown that high levels/activation of the 5' nucleotidase (NT5C2), next to its relevance in AML [157], is also associated with both worse outcomes and cytarabine (AraC) resistance in ALL [158], particular in T-ALL [159,160]. In addition, increased levels of nucleotidase NT5C2 lead to resistance to thiopurines such as 6MP and 6-Thioguanine (6TG) [159,160]. Next to this, also an increased intracellular dCTP pool has been shown to compete with AraC for its incorporation into DNA, thereby effectively outcompeting AraC [161]. As another mechanisms, cytidine deaminase (CDA) is the main inactivating enzyme in the AraC metabolic pathway and is capable to irreversibly deaminate AraC to AraU. High CDA expression levels are associated with AraC resistance [162] and also correlate with increased relapse occurrence in AML [163,164]. Importantly, increased activity of CDA has been shown to protect leukemic cells from AraC induced apoptosis [165,166].

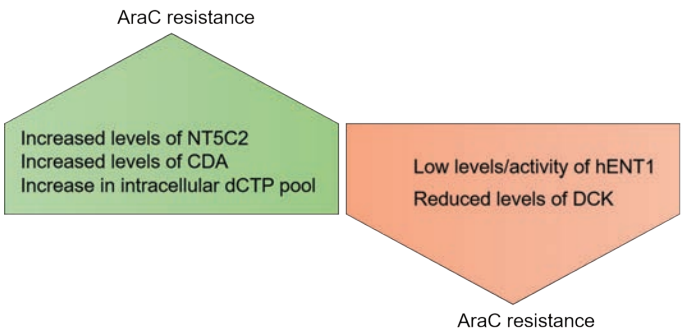


Figure 7 Factors contributing to AraC resistance in leukemia. Abbreviations: Cytosine arabinoside (AraC), human equilibrative nucleoside transporter, (hENT1) Cytidine deaminase (CDA), deoxycytidine kinase (DCK), deoxycytidine triphosphate (dCTP), 5'-Nucleotidase (NT5C2).

Unfortunately, the direct cause of differential chemotherapy responses often remains elusive and there is an urgent need to link genetic events and chemotherapy resistance at a mechanistic level as we still do not understand why specific genetic subgroups respond poorly to therapy in BCP-ALL. To this end, it is necessary to (i) identify novel genetic markers predicting chemotherapy responses and (ii) understand the molecular mechanisms underlying chemotherapy resistance to develop personalized treatment protocols and to open the door for targeted therapy.

4 | Outline of the thesis

Despite the fact that *IKZF1* deletions were identified as a poor prognostic factor, a direct role for *IKZF1* aberrations in chemotherapy resistance was not yet been established at the start of my thesis. Therefore, this thesis aimed to unravel the contribution of the tumor suppressor gene IKZF1 to therapy resistance in BCP-ALL: **Chapter 2** provides an overview of the many faces of the IKZF1 gene by summarizing the impact of IKZF1 on normal and malignant lymphopoiesis and describes signaling pathways affected by loss of *IKZF1*. In **Chapter 3**, we used various model systems to examine the effects of *IKZF1* aberrations on chemotherapy resistance to common chemotherapeutic agents such as GCs, asparaginase and DNA-damaging agents in BCP-ALL. In **Chapter 4**, we identified a potential mechanism responsible for glucocortiocid resistance in *IKZF1*-deleted BCP-ALL. In **Chapter 5**, we have studied if and how other genetic events impact the effects of *IKZF1* on outcome in BCP-ALL patients. We further describe how these co-occurring genetic events contribute to leukemogenesis and therapy resistance in murine models of BCP-ALL. In **Chapter 6**, we investigated whether loss of *IKZF1* influences resistance to other classes of chemotherapeutic agents. Nucleoside analogs, as one of the major classes of antimetabolites used in the treatment of children with leukemia, were of special interest in our studies as IKZF1 has been described to influence the metabolic state of a cancer cell. The work presented in this study is summarized and discussed in **Chapter 7** together with perspectives for future studies. Finally, **Chapter 8** summarizes the work presented in this thesis in Dutch.

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Chapter 2

The many faces of IKZF1 in B cell precursor acute lymphoblastic leukemia

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Abstract

Transcription factor IKZF1 (IKAROS) acts as a critical regulator of lymphoid differentiation and is frequently deleted or mutated in B cell precursor acute lymphoblastic leukemia (BCP-ALL). *IKZF1* gene defects are associated with inferior treatment outcome in both childhood and adult BCP-ALL and occur in more than 70% of *BCR-ABL1* positive and *BCR-ABL1-like* ALL cases. Over the past few years, much has been learned about the tumor suppressive function of IKZF1 during leukemia development and the molecular pathways that relate to its impact on treatment outcome. In this review, we provide a concise overview on the role of IKZF1 during normal lymphopoiesis and the pathways that contribute to leukemia pathogenesis as a consequence of altered IKZF1 function. Furthermore, we will discuss different mechanisms by which *IKZF1* alterations impose therapy resistance to leukemic cells, including enhanced cell adhesion and modulation of glucocorticoid response.

Introduction

B cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common malignancy in children and involves uncontrolled expansion of B-lymphoid progenitors in the bone marrow. The disease is frequently initiated by a chromosomal translocation but becomes manifest only when leukemic progenitors in the bone marrow have accumulated a number of additional gene deletions and mutations that drive disease progression. With current treatment protocols long term survival approaches 90% [1], however relapses still pose a significant clinical challenge due to chemotherapy resistance of the recurrent disease[1]. Both in pediatric and adult BCP-ALL, specific genetic subtypes with distinct prognostic outcomes can be identified [2]. Some of these subtypes, such as hyperdiploid ALL and *ETV6-RUNX1* rearranged ALL are associated with a favorable outcome, while other genetic hallmarks, such as *MLL* gene rearrangements, hypodiploidy, intrachromosomal translocation of chromosome 21 (iAMP21), or the presence of the t(9;22) *BCR-ABL1* translocation predict poor outcome. Moreover, the presence of a gene expression profile similar to *BCR-ABL1* positive ALL, which frequently involves genetic alterations that deregulate cytokine receptor and/or tyrosine kinase signaling, is similarly associated with poor outcome2. In addition to these gross chromosomal rearrangements, deletions or mutations affecting B cell transcription factor *IKZF1*, are a strong and independent predictor of poor outcome in BCP-ALL [3,4]. Next to its role as a critical regulator of B cell development and a leukemia tumor suppressor, there is mounting evidence that IKZF1 loss also affects signaling pathways that modulate therapy response.

Here, we provide an overview of the complex role of transcription factor IKZF1 during normal lymphopoiesis and consequences of IKZF1 loss for BCP-ALL pathogenesis. Finally, we will discuss some of the molecular mechanisms by which *IKZF1* gene alterations may contribute to therapy resistance.

Transcription regulation by IKAROS zinc-finger protein 1

The IKAROS family of transcription factors consists of five different IKAROS zinc-finger proteins (IKZF1-IKZF5) that are able to bind DNA directly at the core motif A/GGGAA through their N-terminal zinc-finger domain [5,6]. Furthermore, all IKAROS family members harbor two additional C-terminal zinc-fingers required for homo- and heterodimerization between the different IKZF proteins (Figure 1A). Formation of homo- or heterodimers between IKAROS zinc-finger proteins with a functional DNA binding domain strongly enhances their DNA affinity and transcriptional activity. However, a common feature of IKZF1 and related family members is the presence of shorter variants due to alternative splicing. These variants often lack DNA binding activity but retain the ability to interact with full-length IKZF1-IKZF5, thereby creating dominant-negative isoforms. A well-known splice variant of both the mouse and human IKZF1 gene is the IK6 isoform, which lacks exons 4 to 7 that encode the four N-terminal zinc-fingers representing the DNA binding domain (Figure 1B).

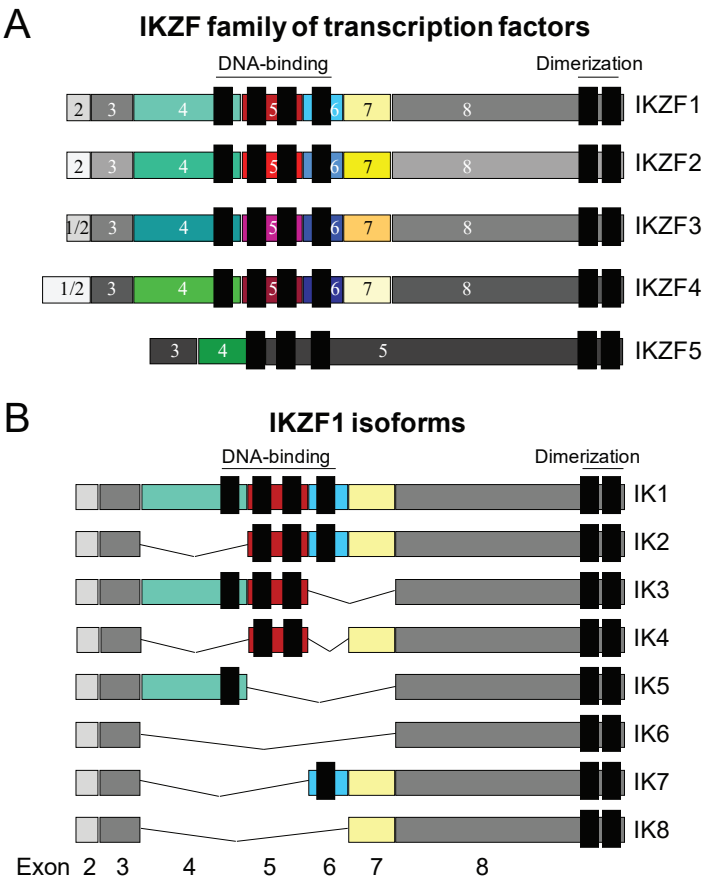


Figure 1 Overview of the human family of IKAROS zinc-finger (IKZF) transcription factors and IKZF1 isoforms. (A) Schematic representation of the five IKZF proteins (IKZF1-IKZF5), including the N-terminal zinc-fingers that define the DNA-binding domain and the two C-terminal zinc-fingers representing the dimerization domain. The colored boxes indicate the individual regions within the protein that are encoded by distinct exons. **(B)** The common IKZF1 splice variants (IK1-IK8) are shown, including the shorter isoforms that are generated by alternative splicing. The splice variants lacking exons 4 and 5 (IK6-IK8) represent dominant-negative isoforms of IKZF1.

IKZF1 mainly regulates gene expression through association with the nucleosome remodeling and deacetylase (NuRD) complex [7-10], which includes histone deacetylases HDAC1, HDAC2 and the ATP-dependent chromatin remodeling proteins CHD3 and CHD4. The NuRD complex is involved in both transcriptional repression as well as gene activation by IKZF1 [11, 12]. Gene silencing by IKZF1 is also facilitated through interaction with Polycomb repressive complex 2 (PRC2), which promotes histone H3 lysine 27 trimethylation (H3K27me3) to maintain genes in an inactive state [13,14]. Other

transcriptional co-factors that can associate with IKZF1 and mediate gene regulation include CtBP, CtIP and SWI/SNF-related complex[15-17]. On the other hand, IKZF1 may itself participate in transcription initiation through direct interaction with the general transcription factors TFIIB and TBP [16]. IKZF1 also controls transcription elongation via association with protein phosphatase 1α (PP1α) and cyclin-dependent kinase 9 (CDK9), the enzymatic component of the positive transcription elongation factor b (P-TEFb) [18-20]. IKZF1-mediated transfer of PP1α to CDK9 promotes P-TEFb activation and recruitment to gene regulatory regions, thereby facilitating transcription elongation of IKZF1-target genes in hematopoietic cells [18].

Distinct post-translational modifications are able to modify the function of IKZF1. Phosphorylation of IKZF1 at multiple serine and threonine residues by casein kinase II (CK2) impairs its function as a transcription factor [20-22]. Conversely, CK2 inhibition enhances the transcriptional repressor function of IKZF1 [23]. On the other hand, dual-specificity kinases BTK and SYK both phosphorylate IKZF1 on specific serine residues in close proximity of the DNA binding domain, to augment its nuclear localization and DNA binding activity [24,25]. Sumoylation of IKZF1 on lysine residues occurs within the nucleus and seems to interfere with transcriptional repression [26,27]. Previously, it was shown that IKZF1 is also subject to ubiquitination [20], but now there is a renewed interest in this pathway, since both IKZF1 and IKZF3 are targets of the immunomodulatory drugs (IMiDs) thalidomide, lenalidomide, pomalidomide and CC-122 [28]. These IMiDs promote proteosomal degradation of IKZF1 and IKZF3 by redirecting the substrate specificity of the $CRL4^{CRBN}$ ubiquitin ligase complex [29,30]. IMiDs show therapeutic effects in a broad range of hematological malignancies by their ability to target the malignant cells and modulate the immune system and its microenvironment.

IKZF1 is essential for normal lymphopoiesis

Studies performed in both constitutive and conditional *Ikzf1* knockout mouse models have demonstrated that IKZF1 function is not only required at different stages of lymphopoiesis [12,31,32], but also for normal myeloid, megakaryocyte and erythroid differentiation [33-36]. *Ikzf1*-deficient mice ($Ikzf1^{null/null}$) lack all B cells, natural killer (NK) cells, plasmacytoid dendritic cells and fetal T cells [31,37] (Figure 2). Nonetheless, post-natal *Ikzf1*-null mice harbor early T lineage progenitors (ETPs) within the thymus and export mature T cells to the periphery [38]. Mice homozygous mutant for a hypomorphic allele of *Ikzf1* ($Ikzf1^{L/L}$) show reduced B cell progenitors in the bone marrow compartment, but still generate normal counts of mature B2 cells [39]. These splenic B cells display alterations in isotype selection during immunoglobulin class switch recombination and a hyper-proliferation phenotype upon antigenic stimulation [40,41]. Although spontaneous progression to B cell acute lymphoblastic leukemia is not observed in $Ikzf1^{L/L}$ mice, haplodeficient $Ikzf1^{L/+}$ animals demonstrate an accelerated onset of B cell leukemia in combination with a BCR-ABL1 transgene [42]. Moreover, all $Ikzf1^{L/L}$ mice develop thymic lymphoma within

a period of 10 months through activation of the Notch pathway [43]. *Ikzf1* mutant mice expressing dominant-negative isoforms of *IKZF1* (*Ikzf1*$^{DN/DN}$ and *Ikzf1*$^{Plstc/Plstc}$) demonstrate a widespread failure of hematopoiesis [44, 45], highlighting the importance of IKAROS transcription factors in hemato-lymphoid differentiation. Notably, heterozygous *Ikzf1* mutant mice develop T cell malignancies with very high penetrance and short latency in case of the dominant-negative isoforms [46, 47], while this phenotype is less obvious in *Ikzf1*$^{+/-}$ mice [48].

Detailed gene expression profiling has revealed that IKZF1 is essential for the generation of common lymphoid progenitors (CLPs) by priming lymphoid lineage-specific signatures in hematopoietic stem cells and lymphoid-primed multipotent progenitors (LMPPs) [49]. At different stages of T-lineage differentiation and development, IKZF1 is engaged by setting thresholds for (pre-)T cell receptor-controlled checkpoints as well as T cell activation downstream of IL-2 receptor signaling [50,51]. In B cell progenitors, *Ikzf1* is required to induce *Rag1* and *Rag2* expression, and mediates chromatin accessibility during immunoglobulin gene rearrangement and allelic exclusion at the *Igk* locus [12,32,52]. During pre-B cell differentiation, IKZF1 regulates the transcription of genes implicated in pre-B cell receptor signaling, cell survival, stromal cell adhesion and B cell commitment, such as *Pax5*, *Foxo1* and *Ebf1* [12,32,53]. Many of those regulatory activities during B-lineage differentiation are navigated by super-enhancer networks controlled by IKZF1 and other B cell master transcription factors [54]. Besides expression regulation of B-lymphoid genes, IKZF1 is actively involved in repression of a lineage-inappropriate transcriptional program normally prevalent in epithelial and mesenchymal precursors [54].

To further delineate the function of the individual zinc-fingers within the DNA-binding domain of IKZF1 in B-lymphopoiesis, *Ikzf1* mouse mutants have been generated with targeted deletion of exon 4, which encodes zinc-finger 1 (*Ikzf1*$^{\Delta F1/\Delta F1}$), or exon 6 encoding zinc-finger 4 (*Ikzf1*$^{\Delta F4/\Delta F4}$) [37]. Germline deletion of either exon 4 or 6 results in decreased B cell precursors with a stronger developmental block in *Ikzf1*$^{\Delta F1/\Delta F1}$ mice, especially at the pre-B cell stage37. In contrast, the fraction of large pre-B cells is strongly increased in *Ikzf1*$^{\Delta F4/\Delta F4}$ mice as compared to wild-type control animals. Interestingly, deletion of zinc-finger 4, but not zinc-finger 1, accelerates the onset of *BCR-ABL1*-mediated B cell leukemia [37,55]. Conditional deletion of exon 5 (*Ikzf1E5*$^{\Delta/\Delta}$), which encodes zinc-fingers 2 and 3, at the CLP-stage also results in an expansion of large pre-B cells within the bone marrow compartment, which is followed by a subsequent block in the transition to small pre-B cells56. These findings indicate that N-terminal zinc-fingers 2, 3 and 4 of IKZF1 limiT cell proliferation and survival at the time of active pre-B cell receptor signaling, while zinc-fingers 1, 2 and 3 are absolutely required for the transition to the pre-B cell stage.

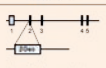
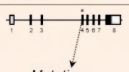
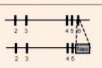
Mouse strain	*Ikzf1*$^{null/null}$	*Ikzf1*$^{L/L}$	*Ikzf1*$^{DN/DN}$	*Ikzf1*$^{Plstc/Plstc}$	*Ikzf1*$^{\Delta F1/\Delta F1}$	*Ikzf1*$^{\Delta F4/\Delta F4}$
Knockout allele	 *Deletion Exon 8*	 *LacZ knock-in Exon 2 (Hypomorphic allele)*	 *Deletion Exon 4-5 (ZF1-3)*	 *Mutation Exon 4 (ZF3) p.H191R*	 *Deletion Exon 4 (ZF1)*	 *Deletion Exon 6 (ZF4)*
B-lymphoid phenotype	• B cells absent	• Mild block B cell development; • Activated B cells; • Disturbed Ig class switch recombination	• B cells absent	• B cells absent	• Block at pre-B cell stage; • Reduction of large pre-BII cells	• Mild reduction progenitor B cells; • Increase of large pre-BII cells
T-lymphoid phenotype	• Fetal T cells absent; • Post-natal T cells activated; • Skewing towards CD4$^+$ lineage	• Normal thymic cellularity; • Activated thymocytes and T cells	• T cells absent	• T cells absent	• Fetal T cells present; • Mild reduction thymic cellularity	• Fetal T cells absent; • Reduced thymic cellularity
Hematopoietic phenotype	• Mild reduction in HSC activity; • NK cells and pDCs absent	• NK cells and pDCs absent; • Increase of neutrophil precursors in fetal liver	• Strong reduction in HSC activity; • Reduction of erythroid progenitors • NK cells and pDCs absent	• Loss of LT-HSC pool at E15.5; • Increase of GMPs at E15.5; • Fatal fetal anemia	• NK cells and pDCs present	• NK cells and pDCs absent
T-lineage malignancies	• Fraction *Ikzf1*$^{null/+}$ mice develops T-cell malignancy	• All *Ikzf1*$^{L/L}$ mice develop thymic lymphoma in 10 mo	• All *Ikzf1*$^{DN/+}$ mice develop thymic lymphoma in 4 mo	• Most *Ikzf1*$^{Plstc/+}$ mice develop T cell malignancy in 4 mo	• None	• All *Ikzf1*$^{\Delta F4/\Delta F4}$ mice develop thymic lymphoma in 10 mo
References	Wang *et al.*,1996[31] Yoshida *et al.*, 2006[33] Avitahl *et al.*,1999[50] Winandy *et al.*,1999[51]	Kirstetter *et al.*,2002[39] Sellars *et al.*,2009[40] Heizmann *et al.*,2016[41] Dumortier *et al.*,2006[43]	Georgopoulos *et al.*,1994[44] Winandy *et al.*,1995[46]	Papathanasiou *et al.*,2003[45] Manta *et al.*, 2007[47]	Schjerven *et al.*,2013[37]	Schjerven *et al.*,2013[37]

Figure 2 Summary of the observed phenotypes in the different constitutive Ikzf1 knockout mouse models. The knockout allele shows a schematic representation at which position the deletion or mutation is present in the mouse *Ikzf1* gene. Abbreviations used: DN, dominant negative; Plstc, ENU-induced dominant-negative point mutation, called Plastic; Neo, Neomycin gene; Geo, fusion between LacZ and Neomycin gene; ZF, zinc-finger; HSC, hematopoietic stem cell; NK, natural killer; pDCs, plasmacytoid dendritic cells; LT-HSC, long term hematopoietic stem cell; GMPs, granulocyte-macrophage precursors; mo, months.

IKZF1 gene lesions drive leukemia development and relapse

In the past decade, complementary genome-wide approaches have been employed to identify the genetic drivers implicated in the pathogenesis of ALL. Those studies revealed that the *IKZF1* gene, which is located on chromosome band 7p12.2, is recurrently affected by different types of genetic alterations in BCP-ALL. Analysis of copy number alterations (CNAs) has demonstrated that *IKZF1* gene deletions are present in about 15% of childhood BCP-ALL cases and 40%-50% of adult patients with BCP-ALL [57-60]. These deletions frequently involve the whole gene (DEL1-8) that results in loss of expression of wild-type IKZF1, as well as focal deletions that alter the function of IKZF1, like the dominant-negative isoform IK6 (DEL4-7). Other common variants include deletions affecting exons 2-3, exons 2-7 and exons 4-8 [61]. In most cases these are monoallelic *IKZF1* deletions where one functional copy of *IKZF1* is retained, although biallelic deletions are also observed in a fraction of BCP-ALL cases [62, 63]. In addition, IKZF1 function is compromised by insertions, frameshift and missense mutations, which represent ~7% of *IKZF1* alterations in BCP-ALL [63]. Furthermore, rare in-frame gene fusions involving IKZF1 have been identified by RNA

sequencing in BCP-ALL, including *IKZF1-NUTM1*, *IKZF1-SETD5* and the reciprocal *SETD5-IKZF1* [64]. However, it remains to be established whether these IKZF1 gene fusions are pathogenic and contribute to leukemia development.

An interesting feature is the strongly increased prevalence of *IKZF1* deletions and mutations in high-risk BCP-ALL cases with an activated tyrosine kinase profile, particularly *BCR-ABL1* positive ALL (~85%) [65], and *BCR-ABL1-like* ALL (~70%), which is characterized by a range of genetic alterations driving cytokine receptor and kinase signaling3, [66-68]. Similarly, *IKZF1* deletions and mutations are highly abundant in chronic myeloid leukemia (CML) that has progressed to lymphoid blast crises, but *IKZF1* alterations are virtually absent in chronic-phase and myeloid blast crises CML [65,69,70]. *IKZF1* deletions are also rarely detected in *ETV6-RUNX1* positive BCP-ALL (3%), TCF3-rearranged (~3%) and *MLL*-rearranged (~5%) B-ALL [58,71,72]. The distribution of *IKZF1* deletions among the remaining subtypes, including hyperdiploid and B-other, ranges from 15%- 20% [72].

IKZF1 acts as a critical tumor suppressor in mouse T-lymphoid malignancies [43,46,47], but *IKZF1* gene lesions are not very prevalent in T-ALL. CNAs and mutations affecting the IKZF1 gene can be detected in ~4% of T-ALL [58,65,71,73]. Notably, *IKZF1* alterations occur in ~13% of early T cell precursor (ETP) ALL, a high-risk T-ALL subtype characterized by recurrent mutations activating tyrosine kinases (*FLT3*, *JAK1*, *JAK3*) and cytokine signaling (*IL7R*) [74]. *IKZF1* alterations have also been reported in myeloproliferative neoplasms [75], and both pediatric and adult acute myeloid leukemia harbor *IKZF1* deletions that affect its function [76,77]. Thus, the tumor suppressive activity of IKZF1 is not uniquely restricted towards the lymphoid lineage and extends to a broader range of hematological malignancies.

Besides its critical role in leukemia pathogenesis, *IKZF1* alterations are also associated with adverse prognosis in BCP-ALL [3,4,78], even within the high-risk group *BCR-ABL1* positive ALL[79, 80]. Notably, the occurrence and prognostic impact of IKZF1 alterations is not restricted to high-risk cases, but is also observed in non-high risk B-ALL subtypes [72], including high hyperploidy81. Indeed, *IKZF1* deletion represents one of the strongest independent predictor of poor treatment outcome in childhood BCP-ALL [71, 72, 82]. Similar data have been reported in adult BCP-ALL, where loss-of-function gene deletions of *IKZF1* predict poor treatment outcome in *BCR-ABL1* negative cases [83-86]. Interestingly, the presence of other co-occurring gene lesions may either enhance or negate the prognostic value of *IKZF1* deletions. For instance, focal deletions affecting both transcriptional regulator *BTG1* and *IKZF1* represent a high-risk group with a worse outcome than *IKZF1* alterations alone48. On the other hand, the BCP-ALL subtype characterized by deregulation of transcription factors *ERG* and *DUX4* has a favorable outcome, despite the strong presence of *IKZF1* deletions in approximately 40% of these patients [64,87-90]. An explanation for this latter observation remains elusive.

Genetic alterations that cooperate with *IKZF1* deletions in BCP-ALL

There is accumulating evidence that recurrent chromosomal aberrations present in BCP-ALL, such as *BCR-ABL1* translocations or *CRLF2* rearrangements, act as driver lesions and represent early events in leukemia development. Genome-wide analysis has established that several other genetic alterations cooperate before B cell leukemia becomes manifest. Especially, gene lesions that inactivate the lymphoid transcription factor IKZF1 are frequently observed in *BCR-ABL1* positive and *CRLF2*-rearranged BCP-ALL [65,69,91,92]. The latter group is associated with concomitant *JAK1* and *JAK2* activating mutations91. Similarly, *IKZF1* alterations are highly prevalent in tyrosine kinase-activating lesions that define *BCR-ABL1-like* ALL [67,68]. These include rearrangements involving *ABL1/ABL2*, *CSF1R*, *EPOR*, *JAK2* and *PDGFRB*, or sequence mutations affecting *FLT3*, *IL7R* or *SH2B3*. Indeed, loss of IKZF1 may permit more effectively STAT5 target gene regulation downstream of these pathways93. Collectively, these findings argue that loss of IKZF1 function strongly cooperates with activated tyrosine kinase signaling pathways linked to enhanced progenitor B cell proliferation and immortalization (Figure 3).

The predilection for *IKZF1* gene alterations in *BCR-ABL1*-mediated lymphoid versus myeloid malignancies has been further corroborated in mouse studies. In a bone marrow transplantation model using lineage-negative hematopoietic progenitor cells, it was shown that expression of IK6 skews *BCR-ABL1*-mediated leukemia from an exclusive myeloproliferative disease towards a combined myeloid and B-lymphoid disease [63]. Introducing $p19^{Arf}$-deficiency further strengthens this trend towards uniformly induced B-ALL. This is in agreement with the finding that *BCR-ABL1* positive BCP-ALL is characterized by the co-occurrence of *IKZF1* and *CDKN2A* gene deletions [65].

Another group of genetic changes that frequently co-occur with *IKZF1* alterations in BCP-ALL include gene deletions affecting lymphoid transcription factors, such as EBF1 and PAX5, and the transcriptional co-factor BTG1 [48,58] (Figure 3). BTG1 belongs to the BTG/TOB antiproliferative (APRO) protein family [94], which control gene transcription by their ability to interact with specific transcription factors, like nuclear receptors and homeobox proteins [95,96], the CCR4-NOT transcriptional regulatory complex [97], or through recruitment of protein arginine methyl transferase PRMT1 [98]. In addition, BTG1 through interaction with the CCR4-NOT, may also regulate mRNA deadenylation and consequently mRNA decay [99, 100]. Mice deficient for *Btg1* show a partial block in B cell development, which is even more evident in $Btg1^{-/-};Btg2^{-/-}$ mice [101]. These studies have demonstrated that BTG1, together with BTG2, is required to suppress a T-lineage inappropriate expression program in progenitor B cells. Thus, mono-allelic gene deletions of *IKZF1* in combination with *EBF1*, *PAX5* or *BTG1* may contribute to a more prominent block in B cell development and increased proliferative expansion of precursor B cells. Indeed, intercrossing haplodeficient *Ikzf1* animals with heterozygous *Ebf1* or *Pax5* knockout mice promotes the onset of acute lymphoblastic leukemia, giving rise to both

B-ALL and T-ALL [102]. On the other hand, Btg1-deficiency specifically accelerates the development of T-ALL in *Ikzf1*$^{+/-}$ mice, which suggests that B-lineage restricted mouse models will be required to establish their synergistic action in B-ALL pathogenesis.

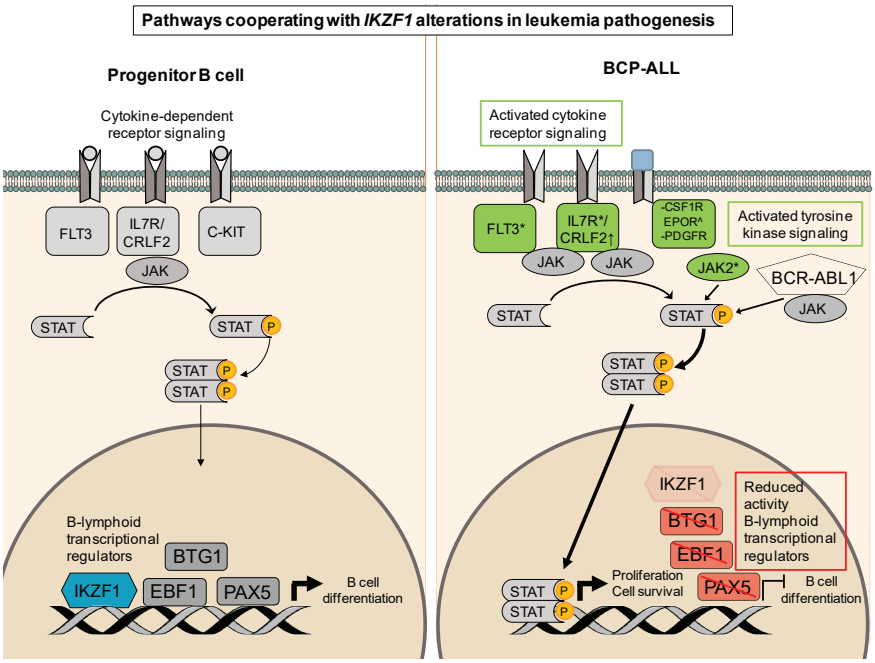


Figure 3 Pathways cooperating with IKZF1 alterations in leukemia pathogenesis. Pathways involving cytokine receptor signaling and B cell differentiation by lymphoid transcriptional regulators in normal progenitor B cells are schematically indicated on the left. Alterations of these pathways co-occur frequently with *IKZF1* deletions and mutations in B cell progenitor acute lymphoblastic leukemia (BCP-ALL) as indicated on the right. These include, activating mutations in FLT3, IL7R, JAK2 (*), upregulation of CRLF2 (□), C-terminal truncations or upregulation of EPOR (^), chromosomal translocations generating fusion proteins with PDGFR or CSF1R (-), and BCR-ABL1, which collectively results in activated cytokine receptor and tyrosine kinase signaling leading to STAT activation. In addition, *IKZF1* alterations co-occur with gene deletions affecting the activity of B-lymphoid transcriptional regulators EBF1, PAX5 and BTG1, which results in a block of B cell differentiation. Abbreviations used: FLT3, FMS Related Tyrosine Kinase 3; IL7R, Interleukin 7 Receptor; CRLF2, Cytokine Receptor Like Factor 2; C-KIT, Mast/stem cell growth factor receptor Kit; JAK, Janus kinase; STAT, signal transducer and activator of transcription; BTG1, B cell Translocation Gene 1; EBF1, Early B cell Factor 1; PAX5, Paired Box 5; IKZF1, IKAROS Family Zinc Finger 1; CSF1R, Colony Stimulating Factor 1 Receptor; EPOR, Erythropoietin Receptor; PDGFR, Platelet Derived Growth Factor Receptor.

Effector pathways downstream of IKZF1 involved in leukemia pathogenesis

Since lymphoid transcription factors are commonly deleted in BCP-ALL, the tumor suppressive functions of IKZF1 and other B cell master regulators, like EBF1 and PAX5, have been mostly linked to the suppression of their B cell differentiation programs in these leukemic cells. However, this would not fully explain the predilection of *IKZF1* alterations in *BCR-ABL1* positive and *BCR-ABL1-like* leukemia, suggesting that IKZF1 regulates also other molecular pathways. Furthermore, loss of IKZF1 function probably affects different target genes in human leukemic cells as compared to mouse progenitor B cells, which could even be distinct from those deregulated by expression of dominant negative isoforms, like IK6. Nonetheless, mouse studies performed over the past 5 years have been very instrumental in deciphering the transcriptional networks downstream of IKZF1. Thus, gene expression profiling in different Ikzf1 knockout mouse models combined with genome-wide chromatin immunoprecipitation studies has uncovered IKZF1-specific targets that are not only linked to lymphoid lineage commitment and B cell differentiation, but also to leukemia development.

A large group of those *Ikzf1*-target genes can be classified as signal transducers, some of which drive early lymphoid differentiation, such as *c-Kit*, *Flt3* and *Il7r* [12,32,37,53]. Adult ALL samples harboring *IKZF1* deletions display increased expression of *IL7R* together with reduced expression of *SH2B3*, which represents a defined subset of high-risk B-ALL [103]. Other genes differentially expressed in *Ikzf1*-mutant mice are important for pre-B cell receptor (BCR) signaling, and several of these IKZF1 targets appear to be deregulated in *BCR-ABL1* positive B-ALL, including *IGLL1*, *SYK*, and *SLP65* [104,105]. Indeed, defective pre-BCR function is a hallmark of *BCR-ABL1* positive ALL, and loss of IKZF1 function enhances SRC phosphorylation at the expense of the SYK/SLP65 pathway activation, which is required for pre-B cell differentiation [104]. Besides transcriptional regulation of signal transducers, *Ikzf1* controls the expression of cell surface receptors, such as *CD34* and *CD43*, and these molecules confer leukemic growth advantage to IKZF1-mutated *BCR-ABL1* positive B-ALL cells [55].

Another group of identified IKZF1 target genes in mouse progenitor B cells seems to converge on a cellular network coupling cell surface protein expression with intracellular Wnt and Rho signaling as well as catenin-driven gene regulation inside the nucleus [55,106]. A critical target gene within this subgroup includes *Ctnnd1* encoding p120-catenin. This is a multifunctional protein that regulates cadherin stability at the cell membrane, activation of Rho family of GTPases in the cytoplasm and Wnt/β-catenin target genes within the nucleus by interacting with Kaiso [107]. Activation of *CTNND1* expression is observed in *IKZF1*-deleted patient samples [108], and inactivation of p120-catenin reduces the proliferative capacity of *BCR-ABL1* positive leukemic cells [55,106]. A related downstream effector pathway of IKZF1 that plays an eminent role during mouse B cell development is integrin-dependent survival signaling, which involves activation of focal adhesion kinase

(FAK) [12,56]. In mouse models of *BCR-ABL1* positive B-ALL, perturbation of *Ikzf1*, including loss-of-function deletions and expression of IK6, leads to activation of an adhesive phenotype, which correlates with overexpression of FAK [63,109]. Focal adhesion pathway upregulation is also observed in *BCR-ABL1* positive BCP-ALL, especially in the context of IK6 expression [109]. Moreover, FAK inhibition potentiates the responsiveness to the ABL inhibitor dasatinib in a xenograft model system and improves survival [109].

Recently, it has been proposed that the B-lymphoid transcriptional program regulated by IKZF1, as well as PAX5, acts as a metabolic barrier against malignant transformation of B cell precursor cells [110]. Inducible reconstitution of functional IKZF1 in patient-derived *IKZF1*-deleted B-ALL cells results in activation of the LKB1-AMPK energy-stress-sensor pathway, and decreased protein levels of the insulin receptor, the glucose transporters GLUT1, GLUT3 and GLUT6, as well as the effectors of glucose metabolism, such as HK2, HK3, and G6PD. On the other hand, the expression of glucose-transport inhibitors, like TXNIP and CNR2, are strongly induced by IKZF1. Consequently, these IKZF1-reconstituted B-ALL cells transit into a state of chronic energy deficit. Thus, this 'metabolic gatekeeper' function of IKZF1 may force silent pre-leukemic clones that carry potentially oncogenic lesions to remain in a latent state.

Besides imposing a change on pre-BCR signaling, cell adhesion and metabolic state, *IKZF1* alterations in combination with BCR-ABL1 expression also result in acquisition of stem cell-like features and enhanced self-renewal of progenitor B cells [63,105] (Figure 4). Activation of THY1 expression has been linked to enhanced self-renewal [63], and *Ikzf1* has been shown to regulate expression of multiple genes involved in cell cycle regulation, including *Cdkn1a*, *Cdkn2a*, and *Cdk6* [53,55]. In mouse progenitor B cells and human B-ALL, *BCL6* and *MYC* have been identified as IKZF1 targets [32,111-113], which both probably contribute to enhanced cell proliferation of *IKZF1*-deleted B-ALL. However, it remains to be established whether targeting of these pathways has therapeutic potential in high-risk B-ALL patients.

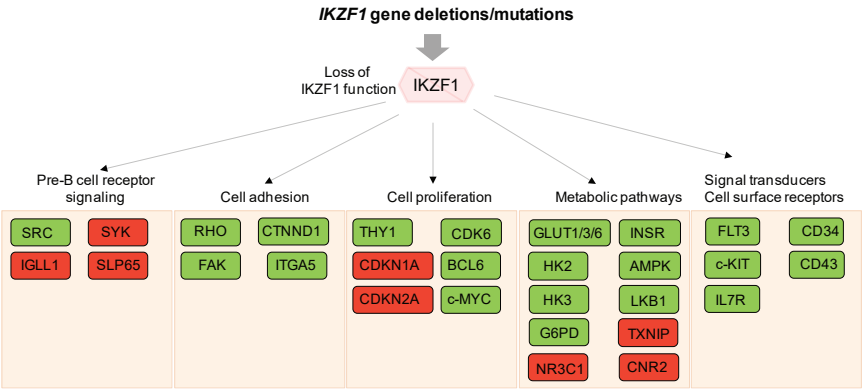


Figure 4 Effector pathways downstream of IKZF1 involved in leukemia pathogenesis. Loss of IKZF1 function due to *IKZF1* gene deletions and mutations affects multiple pathways, including pre-B cell receptor signaling, cell adhesion and proliferation, metabolic pathways and signal transducers and cell surface receptors. IKZF1 affects the expression of defined key molecules within each of these pathways, as indicated in the boxes. Green boxes define targets that are upregulated upon loss of IKZF1 function, while red boxes represent repressed targets. Abbreviations used: SRC, Sarcoma proto-oncogene tyrosine kinase; SYK, Spleen tyrosine kinase; IGLL1, Immunoglobulin lambda like polypeptide 1; SLP65, B cell linker; RHO, RHO family of GTPases ; CTNND1, Catenin delta 1/p120 catenin; FAK, Focal adhesion kinase; ITGA5, Integrin subunit alpha 5; THY1, Thymus cell antigen 1; CDK6, Cyclin dependent kinase 6; CDKN1A, Cyclin dependent kinase inhibitor 1A; BCL6, B cell lymphoma 6; CDKN2A, Cyclin dependent kinase inhibitor 2A; c-MYC, Cellular myelocytomatosis oncogene; GLUT1/3/6, Glucose transporter 1/3/6; INSR, Insuline Receptor; HK2, Hexokinase 2; HK3, Hexokinase 3; AMPK, AMP-activated protein kinase; LKB1, Liver kinase B1; G6PD, Glucose-6-phosphate dehydrogenase; TXNIP, Thioredoxin Interacting Protein; NR3C1, Nuclear Receptor Subfamily 3, Group C, Member 1 (Glucocorticoid Receptor); CNR2, Cannabinoid Receptor 2; FLT3, FMS Related Tyrosine Kinase 3; CD34, Hematopoietic progenitor cell antigen; c-KIT, KIT receptor tyrosine kinase; CD43, Sialophorin; IL7R, Interleukin 7 Receptor.

IKZF1 alterations mediate therapy resistance

The presence of IKZF1 gene lesions in *BCR-ABL1* positive B-ALL result in inferior treatment outcome and mouse xenograft models suggest that IKZF1 loss contributes to resistance to tyrosine kinase inhibitor (TKI)-based therapy [63,80]. Reactivation of cell adhesion pathways by perturbation of IKZF1 function leads to elevation of key adhesion molecules, such as integrins (ITGA5), CD90 and FAK, as well as increased phosphorylation of FAK itself, which permits relocalization of leukemic cells to the bone marrow niche. Indeed, FAK inhibition re-sensitizes *BCR-ABL1* leukemic cells to TKI therapy [109]. Similar results are observed after treatment with retinoids, specifically retinoid X receptor agonists, which induce expression of wild-type IKZF1, but not IK6, thereby abrogating expression of stem cell and adhesion molecules [63]. Although these studies have provided important clues about how *IKZF1* deletions alter treatment response especially in the context of *BCR-ABL1* positive ALL, alternative mechanisms of therapy resistance may exist besides protection through cell interactions within the bone marrow microenvironment.

Synthetic glucocorticoids (GCs), such as prednisolone, constitute essential drugs in the treatment of ALL patients and GC resistance remains a substantial problem in the treatment of BCP-ALL. There is accumulating evidence that *IKZF1* deletions mediate prednisolone resistance *in vivo* [114,115], but different mechanisms have been proposed. IKZF1 actively represses genes of the phosphatidylinositol-3 kinase (PI3K) pathway, including *PIK3CD* and *PIK3C2B* [23]. Disruption of IKZF1 function, and subsequent activation of PI3K/AKT/mTOR pathway can promote GC resistance [116,117]. IKZF1 controls expression of several genes involved in glucose and energy supply [110]. This metabolic program may alter the threshold for responses to glucocorticoids in BCP-ALL. Specifically, the glucocorticoid receptor *NR3C1* was reported as a target of IKZF1 in pre-B ALL cells, and downregulation of NR3C1 protein levels could be observed upon expression of IK6 [110]. However, studies performed in mouse *Ikzf1*$^{+/-}$ B cells and human BCP-ALL cell lines with short hairpin-mediated IKZF1 knockdown have demonstrated that loss of IKZF1 function induces GC resistance independent of altered NR3C1 mRNA and protein expression [114]. Indeed, IKZF1 itself appears to regulate NR3C1-dependent gene transcription [114]. Transcriptional regulator BTG1 has been identified as a modifier of IKZF1-mediated GC therapy resistance and combined loss of BTG1 and IKZF1 leads to even a stronger inhibition of GC-induced cell death48. Finally, IKZF1 target gene *EMP1* [106], which itself represents a poor prognostic factor in pediatric ALL, was shown to regulate the response to prednisolone, but on the other hand also affects normal leukemic cell viability and proliferation [118]. Collectively, these findings demonstrate that IKZF1 through modulation of different signaling pathways and acting directly on GC target genes alters treatment response, thereby mediating therapy resistance in BCP-ALL (Figure 5).

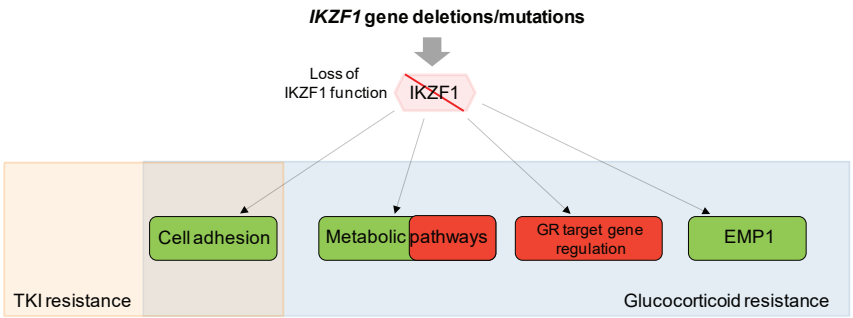


Figure 5 IKZF1 alterations mediate therapy resistance. Overview of IKZF1-affected pathways contributing to tyrosine kinase inhibitor (TKI) resistance and glucocorticoid (GC) resistance. Enhanced cell adhesion due to loss of IKZF1 function has been shown to contribute to both TKI and GC resistance. Deregulation of metabolic pathways, such as LKB1/AMPK signaling and glucose metabolism, attenuated glucocorticoid receptor (GR) target gene regulation and upregulation of epithelial membrane protein 1 (EMP1) have been implicated in mediating GC resistance of *IKZF1*-deleted BCP-ALL. Green boxes indicate activated targets or pathways, while red boxes define attenuated pathways. Targets within the metabolic pathway can either promote or inhibit GC resistance.

Conclusions and perspectives

From this review it becomes clear that loss of IKZF1 function impacts a broad variety of biological pathways which all may contribute to leukemia development. Moreover, the recently established roles for IKZF1 in cell adhesion, metabolism and glucocorticoid-dependent target gene regulation seem to be important determinants of therapy resistance. Preclinical studies are helping with the identification of molecular pathways that can be exploited for targeted therapy of *IKZF1*-deleted BCP-ALL.

Over the past decade, a large series of studies conducted in both childhood and adult ALL have provided clear evidence that *IKZF1* alterations predict adverse outcome in BCP-ALL, both in *BCR-ABL1* positive and negative B-ALL. However, more recently the role of *IKZF1* deletions as an independent prognostic marker has been challenged [119], and also the specific contributions of whole gene versus intragenic dominant-negative *IKZF1* deletions [86]. One potential explanation for such disparities may relate to differences in, scheduling and dosing of specific therapeutic agents between different treatment protocols. Therefore, it will be important to study these protocol-dependent differences in order to define what is currently the most efficient treatment for *IKZF1*-deleted ALL. For the near future, more systematic screens aimed at determining specific vulnerabilities of *IKZF1*-deleted ALL may lead to the identification of targeted therapies that can re-sensitize this high-risk ALL subgroup to curative treatment.

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Chapter 3

Tumor Suppressor IKZF1 Mediates Glucocorticoid Resistance in B cell Precursor Acute Lymphoblastic Leukemia

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Abstract

Synthetic glucocorticoids are essential anticancer drugs in the treatment of acute lymphoblastic leukemia (ALL). Despite improved therapy outcome in ALL, resistance to glucocorticoids remains a major problem in the curative treatment of ALL. Genetic alterations inactivating the transcription factor IKZF1 represent a novel prognostic marker associated with a higher incidence of relapse in patients with B cell precursor ALL (BCP-ALL). In this study, we assessed whether IKZF1 had a direct impact on the sensitivity towards glucocorticoids. Mouse *Ikzf1*$^{+/-}$ B cells were more resistant towards glucocorticoid-induced apoptosis, which correlated with attenuation of the transcriptional response to glucocorticoids. To the converse, IKZF1 overexpression enhanced glucocorticoid receptor-dependent gene transcription in luciferase reporter assay. The glucocorticoid resistance phenotype was also observed in human BCP-ALL cell lines displaying loss of IKZF1 function. In primary BCP-ALL patient samples, *IKZF1* alterations conferred resistance towards prednisolone and dexamethasone as determined by MTT assay, but not other single chemotherapeutic agents. In addition, the frequency of *IKZF1* deletions was significantly higher in patients with a poor prednisolone response *in vivo*. All together, we conclude that tumor suppressor IKZF1 is a key determinant of the cellular response to glucocorticoids in normal and leukemic B cells, and *IKZF1* alterations mediate resistance to glucocorticoids in BCP-ALL.

Introduction

Acute lymphoblastic leukemia (ALL) is characterized by the presence of acquired genetic alterations, including chromosomal translocations, gene deletions and mutations, which drive leukemia initiation and maintenance of the leukemic clone [1,2]. With recent advances in genomic profiling and whole-genome sequencing, the genomic landscape of ALL has been intensively characterized. Several of these genetic alterations were shown to be more prevalent in relapsed leukemia samples and appear to play a direct role in regulating chemotherapy responses, such as mutations in the genes *CREBBP* and *NT5C2* [3-6]. In B cell precursor ALL (BCP-ALL), gene deletions and inactivating point mutations affecting the IKZF1 (IKAROS) gene occur at increased frequencies in high-risk patients harboring a *BCR-ABL1* translocation [7], JAK2 mutation/*CRLF2* rearrangement, and those classified with a Philadelphia- or *BCR-ABL1-like* gene expression signature [10-13]. Moreover, *IKZF1* deletions represent an independent prognostic factor that predicts inferior treatment outcome in different cohorts of childhood and adult BCP-ALL [10,14-17]. However, it has not been established whether loss of *IKZF1* function directly impacts the response to chemotherapy. Synthetic glucocorticoids (GCs), such as dexamethasone and prednisolone, are essential drugs in the multi-agent chemotherapy regimens used to treat patients with lymphoid malignancies, including BCP-ALL. It has been established that both *in vitro* resistance and the initial clinical day 8 response to GCs predict an unfavorable outcome in pediatric ALL [18-20]. GCs act through the glucocorticoid receptor (GR), which is encoded by the NR3C1 gene. Upon binding to the inactive cytoplasmic form of GR, the activated ligand-GR complex translocates to the nucleus and binds to specific DNA sequences known as glucocorticoid response elements (GREs). This results in transcriptional activation of target genes through recruitment of co-activators, like SRC2 [21], or inhibition of gene expression via GR tethering to other transcription factors, GR recruitment of the co-repressor GRIP1, or through binding to negative GREs [22,23]. However, despite intensive research, it remains poorly understood which biological mechanisms contribute to GC resistance phenotype clinically relevant for BCP-ALL.

In this paper, we report that loss of IKZF1 function in normal as well as leukemic B cells confers resistance to GC-induced apoptosis by modulating GR-dependent target gene regulation.

Materials and methods

Ikzf1 knockout animals

In this study, the *Ikzf1*^{Neo} knockout mouse line was used 24, which was kindly provided by M. Busslinger (Research Institute of Molecular Pathology, Vienna, Austria). The *Ikzf1*^{Neo} strain was maintained as a heterozygous knockout line (*Ikzf1*$^{+/-}$) on an inbred *C57BL/6J* genetic background, and all animals were housed under specific pathogen-free conditions.

All animal experiments were approved by the Animal Experimental Committee of the Radboud university medical center and performed in accordance with institutional and national guidelines. For detailed experimental descriptions, see Supplementary methods.

Experimental procedures *Ikzf1* knockout animals

Wild-type and *Ikzf1*$^{+/-}$ mice were sacrificed at the age of 8 to 14 weeks and spleen, tibiae and femurs were removed. The leg bones were flushed to isolate bone marrow (BM) cells and single cell suspensions of splenocytes were obtained using a 70 μM cell strainer. Erythrocytes in the isolates were removed by lysis in Red blood cell lysis buffer (Sigma-Aldrich, Zwijndrecht, Netherlands). After washing the cells, CD19+ Bone marrow (BM) cells were obtained by positive selection on CD19 microbeads according to the manufacturer's protocol (Miltenyi, Bergisch Gladbach, Germany). BM cells were cultured for 48 hours in IMEM medium (Life Technologies, Bleiswijk, Netherlands) supplemented with 25% fetal bovine serum optimized for mouse pre-B lymphoid cells (Stem Cell Technology, Grenoble, France), 0.03% Primaton (Sigma-Aldrich, Zwijndrecht, Netherlands), 1% penicillin/streptomycin (Invitrogen, Merelbeke, Belgium), 50 μM Beta-Mercaptoethanol and 20 ng/mL murine IL-7 (R&D, Oxon, UK). Single cell splenocytes were cultured for 48 hours in RPMI 1640 medium (Life technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% penicillin/streptomycin (Invitrogen), and 50 μM β-Mercaptoethanol in the presence of 5 μg/mL lipopolysaccharide (LPS). Stimulated CD19^{+} BM cells and viable splenic B-lymphocytes obtained after ficoll gradient were subsequently cultured in a 96-well plate at a density of 1x 105 cells/well under similar conditions as the initial expansion phase. B cells were treated with increasing concentrations of prednisolone or dexamethasone (both Centrafarm, Etten-Leur, the Netherlands). After 48 hours, relative cell viability was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation (MTS) Assay (Promega, Madison, WI). Absorbance was acquired using a plate reader (Infinite F50; TECAN, Männedorf, Switzerland). Phenotypic characterization of the B-lymphocytes derived from WT and *Ikzf1*$^{+/-}$ mice after 48h exposure to 5 μg/mL LPS was obtained by B220 and CD19 (both BD Pharmingen, San Jose, CA) staining and analyzed by FACS using the LSRII flow cytometer (BD Biosciences, San Jose, CA). For AnnexinV analyses splenic B-lymphocytes derived from 8-14 weeks old WT and *Ikzf1*$^{+/-}$ mice were treated for 48h with prednisolone or dexamethasone and stained with the AnnexinV-PE/7-AAD Viability Kit (BD Biosciences) according to manufacturer's instructions and analyzed by FACS. AnnexinV positive cell fractions were determined by FlowJo software Version 5.03 (Treestar, Ashland, OR). For analysis of PARP cleavage, cleaved PARP fractions were divided by uncleaved PARP fractions to determine the relative PARP cleavage ratio.

Protein lysates and immunoblotting

Whole cell extracts were prepared in NP-40 lysisbuffer (200mM NaCl, 0.5% NP-40, 20mM Tris.Cl pH8.0, 5mM EDTA, 1mM Na3VO4, 100mM NaF) supplemented with 1mM PMSF and Complete protease inhibitors (Roche Life Sciences, Almere, Netherlands), and

cleared by centrifugation. Protein concentration was determined with Biorad assay and equal amount of protein lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were probed with primary antibodies against PARP (# 9542), GR (#12041), pSer211-GR (#4161) (Cell Signaling Technology, Leiden, the Netherlands), GAPDH (H-12, Santa Cruz Biotechonology, Santa Cruz, CA), IKZF1 (R&D Systems Oxon, UK), followed by horseradish peroxidase (HRP) conjugated secondary antibody. Proteins were visualized with ECL reagent (GE Healthcare) and expression was detected with Fluorchem (Cell biosciences, Santa Clara, USA). Quantification was performed with AlphaView software version 3.3.10 (Cell biosciences, Santa Clara, USA).

Microarray-based gene expression analyses

Total RNA was extracted from 3x106 splenic B cells from WT (*n*=3) and *Ikzf1*$^{+/-}$ mice (n=3) according to the manufacturer's protocol (Qiagen RNAeasy kit, Venlo, the Netherlands). The RNA of each group was pooled and biotinylated complementary RNA was prepared using the Illumina TotalPrep RNA Amplification kit (Ambion, Austin, TX) according to manufacturer's specifications with an input of 200ng total RNA. Samples were hybridized onto the Illumina MouseRef-8 v2 at ServiceXS B.V (Leiden, The Netherlands). Samples were normalized to Robust Multi-array Average (RMA) and analyzed with ArrayStar software (DNAStar, Madison, WI). We then used the Ingenuity software package to conduct upstream regulator analyses of the genes that were differentially expressed between untreated and prednisolone-treated WT and *Ikzf1*$^{+/-}$ B cells with an absolute value expression cut-off of 3.5 and a fold change (FC) ≥ 2. Illumina bead array expression data are available at the NCBI Gene Expression Omnibus database (GSE65554).

Real-time quantitative polymerase chain reaction

Total RNA was extracted using the RNeasy mini-kit (Qiagen). Subsequently, cDNA was synthesized of 500ng RNA template using the iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA). mRNA expression levels were determined by use of Power SYBR® Green PCR master mix using gene-specific primers (sequences are listed in Supplementary Table 1) and the CFX96 TouchTM Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). HPRT mRNA expression was used as a reference to obtain the relative fold expression of target genes using the comparative cycle threshold $2^{(-\Delta\Delta Ct)}$ method.

Luciferase reporter assay

A minimal mouse mammary tumor virus (MMTV)-Luc promoter construct was generated by cloning a 405-bp region containing four glucocorticoid response elements (GREs) of the long terminal repeat (LTR) region of MMTV into the *NheI-XhoI* restriction sites of the pGL3-basic plasmid vector (Promega, Madison, WI, USA). HEK293 cells were cultured in DMEM medium with 10% FCS and 1% Pen/Strep in 24-well plates and transfected with

MMTV-Luc promoter reporter construct, plasmid human influenza hemagglutinin (pHA)-N1-GRalpha expression vector, pcDNA-StrepII-FLAG-Ty1-IKZF1 or pcDNA-StrepII-FLAG-Ty1-IK6 expression plasmids using polyethylenimine (Pei) reagent. A cytomegalovirus immediate early promoter (CMV)-Renilla expression plasmid was included to control for transfection efficiency. After transfection, cells were treated with 3 μM prednisolone for 20 hours before cells were harvested with Passive Lysis Buffer (Promega Madison, WI). Luciferase activity was determined by using the dual luciferase reporter assay system following the manufacturer's instructions (Promega Madison, WI).

Lentiviral shRNA-mediated gene knockdown and lenalidomide treatment

RS4;11 and NALM6 leukemia cell lines were cultured in RPMI160 medium (Life Technologies, Bleiswijk, Netherlands) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Invitrogen, Merelbeke, Belgium). For lentiviral transduction, HEK293FT cells were transfected with one lentiviral pLKO.1-puro vector (see Supplementary Table 1 for shRNA sequences) together with pVSV-G and psPAX2 using Lipofectamine 2000 (Invitrogen). Leukemia cell lines were transduced in the presence of 1 μg/mL polybrene and 24 hours after transduction selected with 1μg/mL puromycin. For lenalidomide-induced IKZF1 protein degradation, RS4;11 were pretreated with 1μg/ml lenalidomide (Selleckchem, Houston, TX, USA) for 24 hours. Subsequently, cells were in continuous presence of 1μg/mL lenalidomide during incubation with prednisolone or dexamethasone for MTS assays and AnnexinV/7AAD assays (48 hours) or RNA isolation (16 hours).

Patient leukemia samples and MTT assay

The MTT data were obtained from 187 diagnosis samples of the Dutch Childhood Oncology Group (DCOG) trial ALL-9. The day 8 prednisolone response was obtained from 646 patients of ALL-10 and ALL-11 trials. In accordance with the Declaration of Helsinki, written informed consent was obtained from parents and guardians, to use excess diagnostic material for research purposes. Viable leukemic cells were obtained from fresh bone marrow or peripheral blood samples using a ficoll gradient. The mononuclear cells were cultured in 96-well plate at a density of 1.6 x 10^5 cells/well in complete culture medium, which consisted of RPMI 1640 medium supplemented with 20% heat-inactivated FCS, 2 mM Glutamine, 200 g/mL Gentamycine, 5 μg/mL Insuline, 5 μg/mL Transferrine, and 5 ng/mL Sodium selenite. The following chemotherapeutic drugs were added to the cells in duplicate in 6 different concentrations: L-Asparaginase /Paronal (Takeda Pharmaceuticals, Zurich, Switzerland), range 0.0032-10 U/mL; dexamethasone (Brocacef, Maarssen, the Netherlands), range 0.0005-15 μM; vincristine/Oncovin (Eli Lilly, Indianapolis, In), range 0.06-60 μM; prednisolone (Brocacef, Maarssen, the Netherlands), range 0.02-700 μM. After 4 days, 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) was added to the cells. After 6 hours incubation at 37 °C, isopropanol-HCl was added and samples were measured in microplate reader at 562 nM wavelength. To

detect *IKZF1* deletions and mutations, genomic DNA was isolated from leukemic blasts present in bone marrow or peripheral blood with the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. *IKZF1* gene deletions were identified using the SALSA P335 ALL Multiplex Ligation-dependent Probe Amplification (MLPA) assay (MRC-Holland, Amsterdam, the Netherlands). *IKZF1* deletions were confirmed with the previously described P202 MLPA probe set. The presence of inactivating point mutations in *IKZF1* was determined using PCR amplification and subsequent Sanger sequencing of exons 4, 5, 6 and 8.

Statistical analyses

The statistical analyses for the cell viability assays and quantitative real-time polymerase chain reaction (qRT-PCR) were performed using PRISM6 (GraphPad Software, La Jolla, CA), and for the MTT data of the patient samples, the SPSS program (IBM, Amsterdam, Netherlands) was used. For the MTS assays, two-sided ANOVA was performed to assess differences between the best-fit curves. For the AnnexinV, PARP cleavage and qRT-PCR, a student's *t* test was performed. For the patient data (MTT assay), the statistical significance in the *IKZF1*-Mut group versus *IKZF1*-WT group was calculated using the Mann-Whitney U test. All statistical tests were two-sided and *P* values of less than 0.05 were considered statistically significant.

Results

Glucocorticoid resistance phenotype in *Ikzf1*-haplodeficient B cells

To address whether loss of the transcription factor IKZF1 has an impact on the level of GC-induced apoptosis in lymphoid cells, we analyzed the phenotype of mouse B cells lacking one functional allele of *Ikzf1*. Lipopolysaccharide (LPS)-activated splenic B cells obtained from *Ikzf1*$^{+/-}$ mice showed enhanced cell survival after treatment with GCs compared to wild-type (WT) cells as measured with MTS assay (Figure 1a), which was not related to differences in B cell differentiation (Supplementary Figure S1). The inhibitory concentration targeting 50% of the cells (IC_{50}) after prednisolone treatment was about 90-fold higher in *Ikzf1*$^{+/-}$ B cells compared to wild type control cells ($P<0.001$) (Figure 1a, left panel). An even stronger effect was observed for dexamethasone, with a 275-fold higher IC_{50} for *Ikzf1*$^{+/-}$ B cells ($P<0.001$) (Figure 1a, right panel). A similar phenotype was observed in bone marrow-derived CD19^{+} progenitor B cells, where *Ikzf1*-haplodeficiency also conferred resistance to GC-induced apoptosis (Supplementary Figure S2). The resistance against GC-induced apoptosis in splenic B cells was confirmed by AnnexinV staining, where the fraction of AnnexinV-positive cells was significantly lower in *Ikzf1*$^{+/-}$ B-lymphocytes treated with prednisolone or dexamethasone, compared to WT B-lymphocytes ($P<0.001$ for both drugs) (Figure 1b, Supplementary Figure S3). In addition, another marker of apoptosis, PARP protein cleavage, revealed that *Ikzf1*$^{+/-}$ B cells displayed

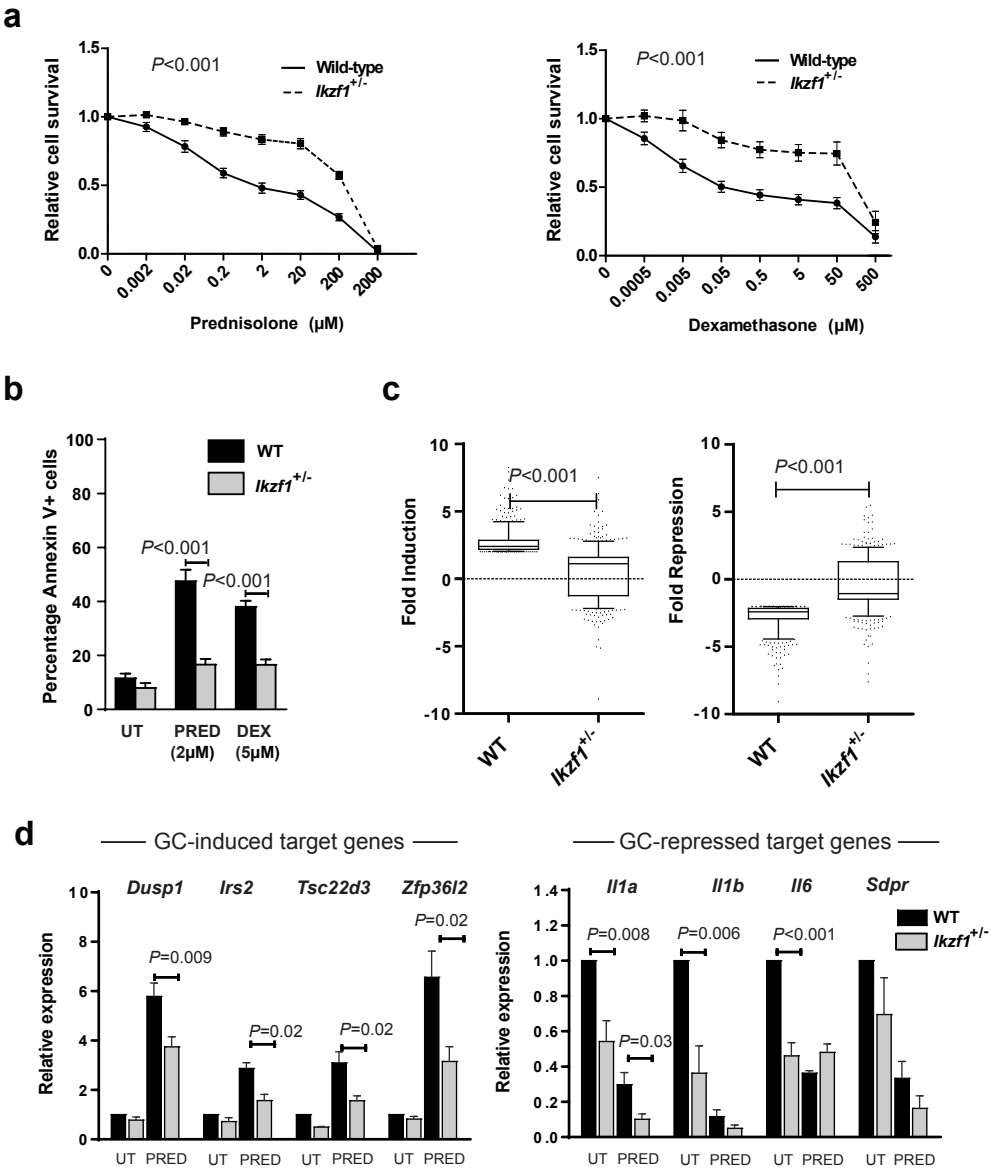
lower levels of cleaved PARP after prednisolone treatment in comparison to WT control cells (Supplementary Figure S4). Together these data demonstrate that IKZF1 is a critical determinant of GC-induced apoptosis in B cells.

Attenuated glucocorticoid-responsive target gene regulation in *Ikzf1*-haplodeficient B cells

To explore the mechanism underlying the glucocorticoid resistance phenotype, we performed microarray gene expression analyses on wild-type and *Ikzf1*$^{+/-}$ B-lymphocytes. Ingenuity upstream regulator analyses revealed a dexamethasone-related signature in both untreated and prednisolone-treated B cells (Supplementary Figure S5, Supplementary Table 2), indicating that GC-regulated target genes were differentially expressed in *Ikzf1*$^{+/-}$ B cells. Indeed, the level of gene induction of 769 prednisolone-activated genes in *Ikzf1*$^{+/-}$ B-lymphocytes was significantly lower compared to the WT cells ($P<0.001$; Figure 1c, left panel). Similarly, the 774 prednisolone-repressed genes showed a significantly impaired response in *Ikzf1*$^{+/-}$ B-lymphocytes compared to controls ($P<0.001$; Figure 1c, right panel). Comparison with the global gene expression signature derived from *IKZF1*-deleted BCP-ALL patient samples showed a minimal overlap (Supplementary Figure S6).

To further investigate the expression of individual GC-responsive genes, quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed for seven genes known to be activated by GCs. The transcriptional response of *Tsc22d3*, *Dusp1*, *Irs2* and *Zfp36l2* was significantly attenuated by *Ikzf1* haplodeficiency (Figure 1d), whereas three other GC-activated genes (*Sgk1*, *Ddit4*, and *Fkbp5*) were not differentially regulated in response to prednisolone (Supplementary Figure S7). All four GC-repressed target genes examined (*Il1a*, *Il1b*, *Sdpr*, *Il6*) displayed a lower basal level of expression in *Ikzf1*$^{+/-}$ B-lymphocytes, of which only one (*Il6*) displayed an attenuated response to prednisolone. (Figure 1d). Together, our results show that in *Ikzf1*$^{+/-}$ B cells a major subset of GC-responsive target genes display lower steady-state expression levels and significantly impaired gene regulation by prednisolone.

Figure 1 Glucocorticoid-resistance phenotype in *Ikzf1*$^{+/-}$ B cells. **(a)** Wild-type (WT) and *Ikzf1*$^{+/-}$ splenic B cells pre-stimulated by lipopolysaccharide (LPS) were treated for 48 hours with increasing concentrations of prednisolone (left panel) or dexamethasone (right panel) and analyzed by MTS assay (n=9). All values were normalized to untreated B cells. Error bars represent ± standard error of the mean (SEM). P values were calculated based on the differences of the best-fit curve. **(b)** AnnexinV/7-AAD staining was performed on WT and *Ikzf1*$^{+/-}$ B cells after 48 hours prednisolone (n=6) or dexamethasone (n=5) treatment and analyzed by flow cytometry. Data are means and SEM. **(c)** Boxplots indicate the median level of 769 prednisolone-activated target genes (left panel) or 774 prednisolone-repressed target genes (right panel) in wild-type B cells as compared to the level of target gene regulation in *Ikzf1*$^{+/-}$- B cells. **(d)** Transcript levels of glucocorticoid (GC)-target genes that were either activated (left panel) or repressed (right panel) by prednisolone treatment as measured by quantitative reverse transcription polymerase chain reaction in B cells from WT (n=4) and *Ikzf1*$^{+/-}$ mice (n= 4). All expression levels were normalized to Hprt expression and compared to the levels in the untreated (control) WT B cells. Data are means and SEM.


a
Relative cell survival
P<0.001
Wild-type
Ikzf1+/-
Prednisolone (μM)
Dexamethasone (μM)
b
Percentage Annexin V+ cells
WT
Ikzf1+/-
P<0.001
UT
PRED (2μM)
DEX (5μM)
c
Fold Induction
Fold Repression
d
GC-induced target genes
GC-repressed target genes
Dusp1
Irs2
Tsc22d3
Zfp36l2
Il1a
Il1b
Il6
Sdpr
Relative expression
P=0.009
P=0.02
P=0.008
P=0.006
P=0.03

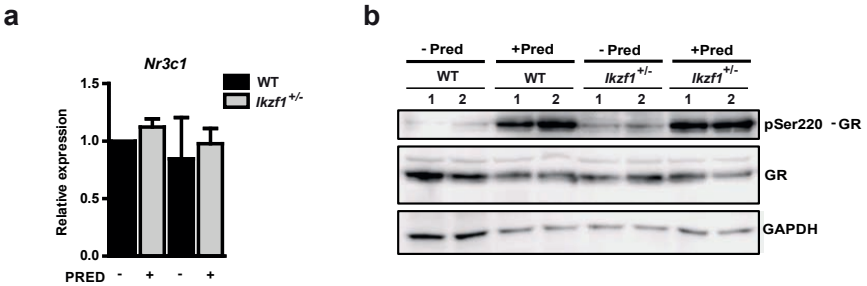


Figure 2 Expression and phosphorylation of glucocorticoid receptor in I*Ikzf1*$^{+/-}$ B cells in response to glucocorticoid treatment. **(a)** Expression levels for *Nr3c1* were analyzed in wild-type (WT) and *Ikzf1*$^{+/-}$ B cells (both *n*=3) by quantitative reverse transcription polymerase chain reaction after 16 hours of 2μM prednisolone treatment and normalized to *Hprt* expression. Data are means and SEM. **(b)** Immunoblot shows levels of phospho-Ser220 and total levels of glucocorticoid receptor (GR) in mouse B cells of two independent WT (WT1, WT2) and *Ikzf1*$^{+/-}$ (*Ikzf1*$^{+/-}$-1 and *Ikzf1*$^{+/-}$-2) animals in the absence or presence of 16 hours 2μM prednisolone treatment. GAPDH antibody staining serves as a loading control.

Glucocorticoid-dependent signaling in *Ikzf1*-haplodeficient B cells

Different aspects of GC signaling could be responsible for the altered state of GC-mediated gene regulation in *Ikzf1*$^{+/-}$ B cells, including indirect effects on the expression or activity of the glucocorticoid receptor (GR). To investigate this possibility, we first determined whether basal mRNA expression and auto-induction of the GR gene *Nr3c1* itself was affected by Ikzf1 levels, which was not the case (Figure 2a). Next, the level of GC-induced phosphorylation of GR was investigated. It has been well established that in response to GCs, cyclin-dependent kinases (CDKs), glycogen synthase kinase 3 (GSK-3), and mitogen activated protein kinases (MAPKs) can phosphorylate GR at multiple sites [25-27], including serine residue 220 of mouse GR. Western blot analysis showed similar levels of prednisolone-induced phosphorylation on serine 220 (pSer220) in B-lymphocytes of two independent WT and *Ikzf1*$^{+/-}$ mice (Figure 2b). Together, these data indicate that IKZF1 has no significant impact on the expression and activation of GR in primary B-lymphocytes treated with prednisolone.

Effect of IKZF1 on glucocorticoid receptor-dependent transcription

Next, we assessed more direct effects of IKZF1 on GR-dependent gene transcription. To this end, a GC-responsive luciferase reporter construct was used, harboring a minimal MMTV promoter fragment with four glucocorticoid responsive elements (GREs). Both full length IKZF1 and the dominant negative deletion variant IK6, which can be detected in BCP-ALL patients with intragenic *IKZF1* deletions [28], were tested. HEK293 cells transfected with IKZF1 and GR expression plasmids displayed an IKZF1 concentration-dependent activation of GR-induced gene transcription in the presence of prednisolone

(Figure 3a). This additional increase of luciferase activity by IKZF1 was not observed in the absence of transfected GR plasmid (Figure 3a). Moreover, transfection of similar levels of IK6 had no effect on GR-mediated transcriptional activation (Figure 3a). Western blot analyses confirmed that IKZF1 had no impact on GR protein levels (Figure 3b), suggesting that IKZF1 activates GR-dependent transcription.

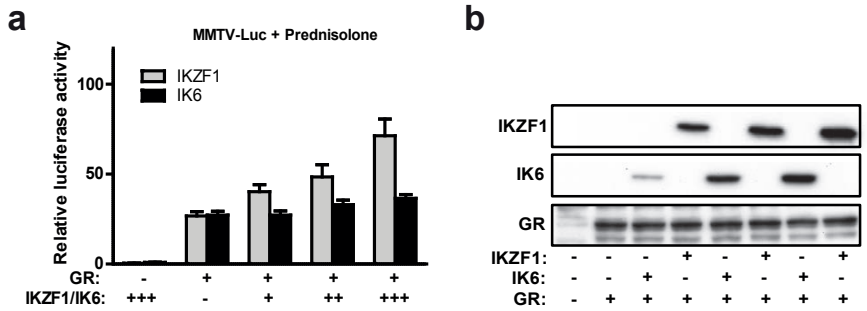


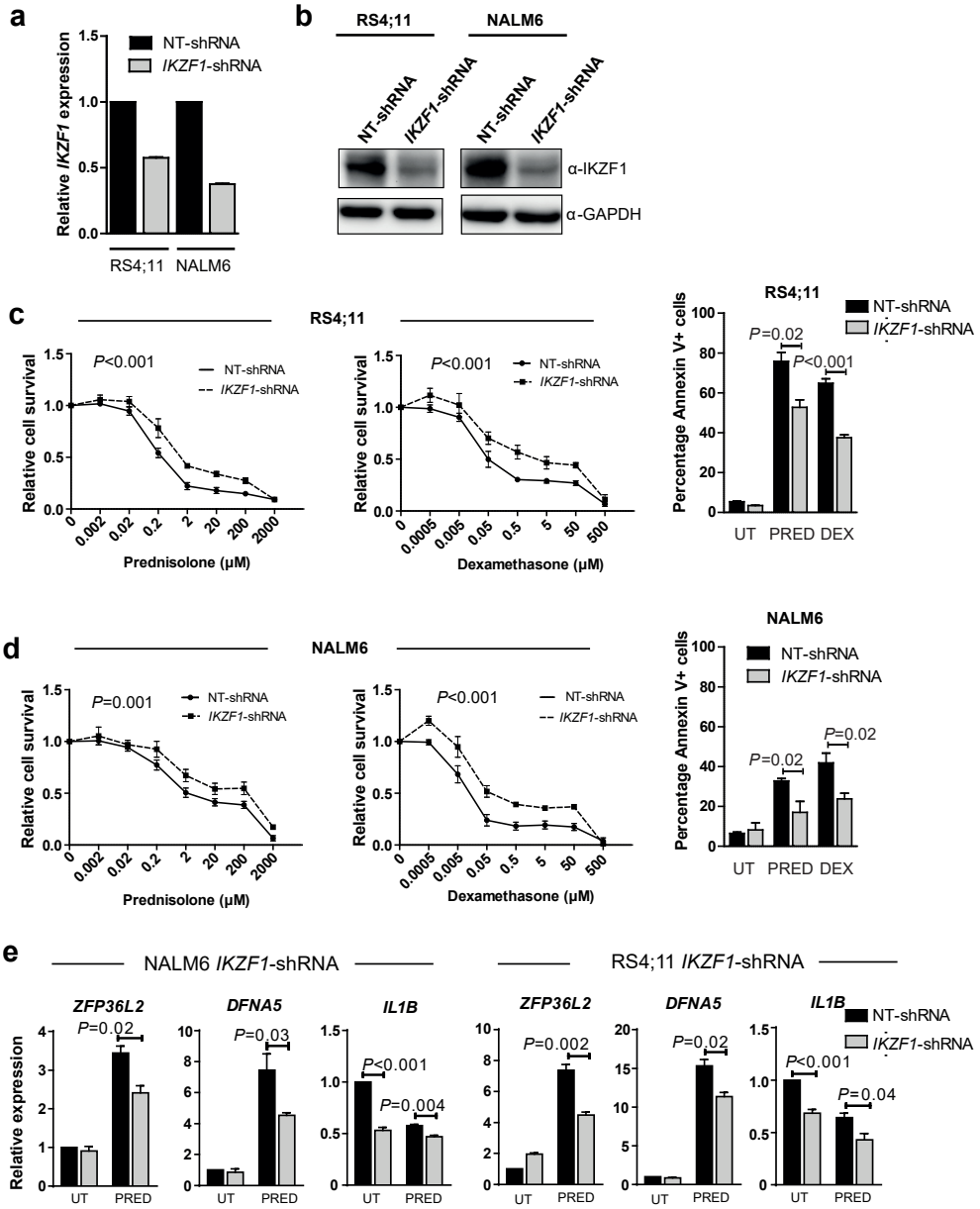
Figure 3 IKZF1 overexpression enhances glucocorticoid receptor-dependent gene activation. **(a)** Luciferase reporter assay was performed in HEK293 cells by co-transfection of a MMTV-Luc reporter construct harboring four glucocorticoid responsive elements together with expression plasmids for HA-tagged glucocorticoid receptor (GR), full length IKZF1 or mutant IK6 lacking amino acids 54-283, including the four N-terminal zinc-fingers encoding the DNA binding domain, and CMV-Renilla. Normalized luciferase activity for each sample was related to MMTV-Luc vector alone. Results represent the mean and standard deviation from two independent experiments performed in duplicate. **(b)** Western blot analysis shows expression levels of IKZF1, IK6 and GR as used for the MMTV-Luc reporter assay using IKZF1 (for IKZF1 and IK6 detection) and HA (for GR detection) antibodies

Silencing of IKZF1 expression induces glucocorticoid resistance in leukemia cell lines

We next determined whether loss of IKZF1 function would also alter the response to GCs in the BCP-ALL cell lines RS4;11 and NALM6, using lentiviral-mediated short hairpin RNA (shRNA) approach. *IKZF1*-shRNA expression resulted in a ~50% reduction in IKZF1 mRNA and protein expression compared to non-targeting (NT)-shRNA (Figure 4a-b). MTS assays revealed that *IKZF1*-silencing inhibited GC-induced apoptosis in RS4;11 cells (Figure 4c), where the IC_{50} for prednisolone was ~10-fold higher in the *IKZF1*-shRNA cells compared to NT-shRNA cells ($P<0.001$). For dexamethasone, the IC_{50} was ~30 fold higher in the *IKZF1*-shRNA transduced RS4;11 cells compared to NT-shRNA cells ($P<0.001$). Similar results were obtained in NALM6 cells, where the IC_{50} for prednisolone and dexamethasone was significantly higher in comparison to NT-shRNA cells ($P<0.001$) (Figure 4d). The GC resistance phenotype was confirmed with AnnexinV staining in both cell lines, showing enhanced survival for *IKZF1*-shRNA transduced cells in response to GCs (Figure 4c-d, right panels). The GC-resistance phenotype was shown to correlate with attenuated

regulation of prednisolone-responsive target genes *ZFP36L2*, *DFNA5* and *IL1B* in both RS4;11 and NALM6 cells transduced with IKZF1-shRNA (Figure 4e). In a second approach, the thalidomide analog lenalidomide was used to selectively degrade IKZF1 protein in RS4;11 cells [29,30]. Treatment for 24 hours with 1-2μM lenalidomide significantly reduced IKZF1 protein levels in RS4;11 cells (Figure 5a). Lenalidomide-treated RS4;11 cells also displayed a protection against GC-induced apoptosis as measured by MTS assay (Figure 5b) and AnnexinV staining (Figure 5c). This Lenalidomide-induced GC resistance phenotype observed in RS4;11 could also be confirmed in ALL-PO (Supplementary Figure S8). Similar to the shRNA approach, lenaldiomide-treated RS4;11 cells demonstrated altered regulation of GC-responsive genes (Figure 5d), including attenuation of the GC-induced target genes *SGK1*, *DFNA5* and *FBXW7*, as well as decreased basal levels of the GC-repressed target genes *PRICKLE1* and *IL1B*. Together, these data indicate that loss of IKZF1 function confers resistance towards glucocorticoids in human BCP-ALL cell lines by affecting the glucocorticoid transcriptional response.

Figure 4 Effect of IKZF1 silencing on glucocorticoid resistance phenotype in leukemia cell lines. (a-b) Expression analysis of IKZF1 in BCP-ALL cell lines RS4;11 and NALM6 lentiviral transduced with NT- and *IKZF1*-shRNA. **(a)** Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to assess the efficiency of IKZF1 silencing in RS4;11 and NALM6 cell lines. Relative expression levels of *IKZF1* knockdown were compared to non-targeting (NT) shRNA. **(b)** Western blot analysis of IKZF1 protein levels in NT-shRNA and *IKZF1*-shRNA expressing cell lines. GAPDH was used as loading control. **(c-d)** MTS assay (left panels) or AnnexinV staining (right panels) were performed on RS4;11 (c) and NALM6 **(d)** cells expressing NT-shRNA or *IKZF1*-shRNA 48 hours after treatment with increasing concentrations of prednisolone or dexamethasone. All values were normalized to NT-shRNA for MTS analysis. Error bars represent ± standard error of the mean (SEM) from three independent experiments. For MTS assays, *P* values were calculated based on the differences of the best-fit curve. For AnnexinV/7AAD assays, data represent the means from three independent experiments, and error bars represent SEM. **(e)** Relative expression of commonly shared glucocorticoid (GC)-responsive target genes *ZFP36L2*, *DFNA5* and *IL1B* in RS4;11 and NALM6 cells expressing *IKZF1*-shRNA. GC-target gene expression was measured after 16 hours 2μM prednisolone treatment as determined by qRT-PCR and normalized to *HPRT* expression. All expression levels are compared to untreated (UT) NT-shRNA cells. Error bars represent SEM from at least three independent experiments.


a
Relative IKZF1 expression
NT-shRNA
IKZF1-shRNA
RS4;11
NALM6
b
RS4;11
NALM6
NT-shRNA
IKZF1-shRNA
α-IKZF1
α-GAPDH
c
RS4;11
P<0.001
Relative cell survival
Prednisolone (μM)
Dexamethasone (μM)
Percentage Annexin V+ cells
P=0.02
UT PRED DEX
d
NALM6
P=0.001
e
NALM6 IKZF1-shRNA
RS4;11 IKZF1-shRNA
ZFP36L2
DFNA5
IL1B
Relative expression
P=0.02
P=0.03
P<0.001
P=0.004
P=0.002
P=0.04
UT
PRED

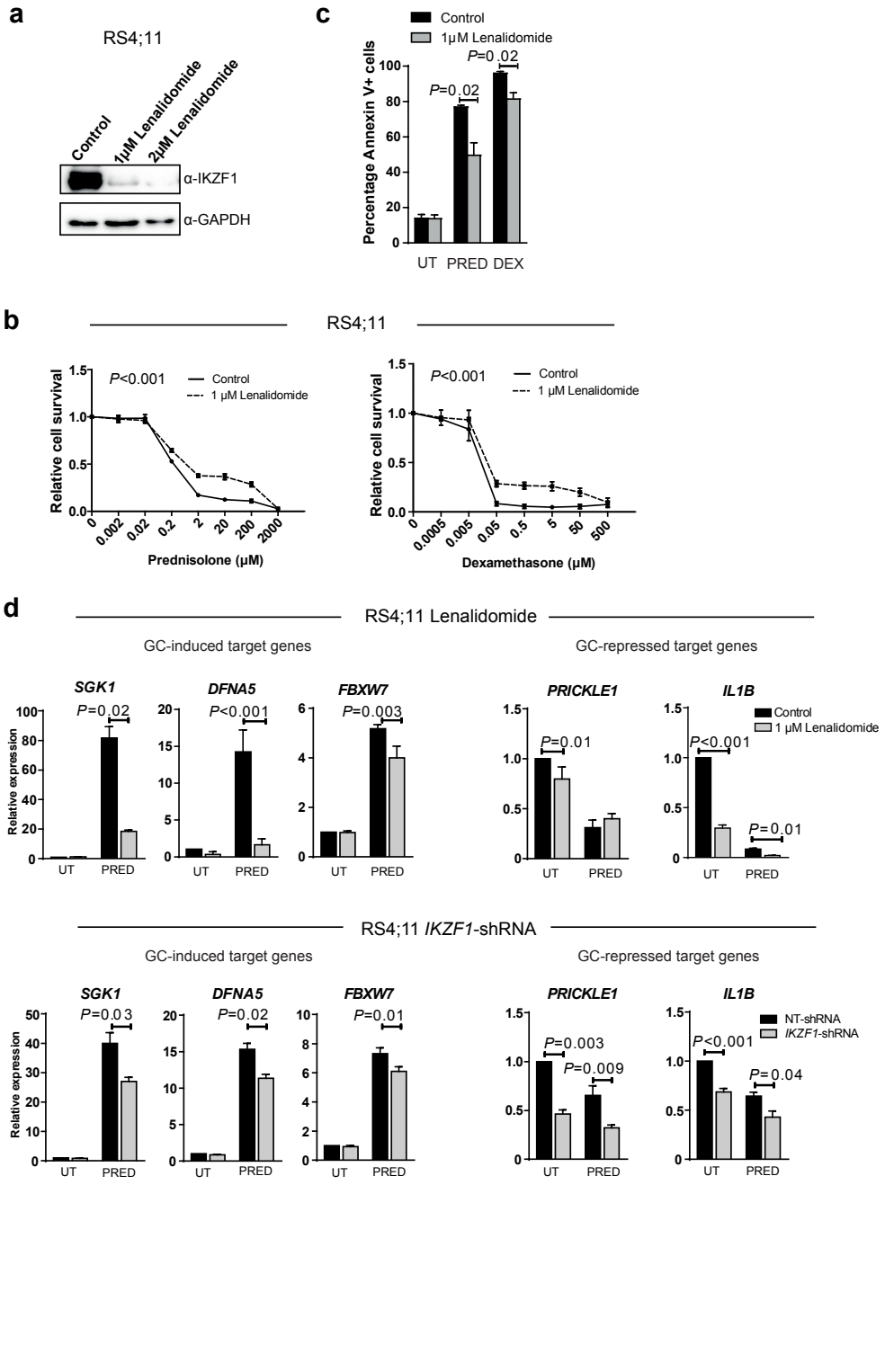
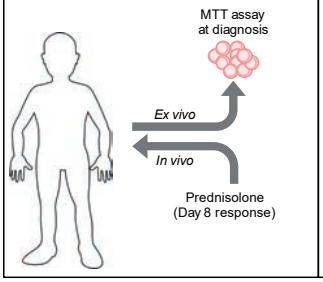

a
RS4;11
Control
1μM Lenalidomide
2μM Lenalidomide
α-IKZF1
α-GAPDH
c
Control
1μM Lenalidomide
Percentage Annexin V+ cells
P=0.02
P=0.02
UT PRED DEX
b
RS4;11
P<0.001
Control
1 μM Lenalidomide
Relative cell survival
Prednisolone (μM)
P<0.001
Control
1 μM Lenalidomide
Relative cell survival
Dexamethasone (μM)
d
RS4;11 Lenalidomide
GC-induced target genes
GC-repressed target genes
SGK1
P=0.02
DFNA5
P<0.001
FBXW7
P=0.003
PRICKLE1
P=0.01
IL1B
P<0.001
P=0.01
Control
1 μM Lenalidomide
Relative expression
UT PRED
RS4;11 IKZF1-shRNA
GC-induced target genes
GC-repressed target genes
SGK1
P=0.03
DFNA5
P=0.02
FBXW7
P=0.01
PRICKLE1
P=0.003
P=0.009
IL1B
P<0.001
P=0.04
NT-shRNA
IKZF1-shRNA
Relative expression
UT PRED

Figure 5 Lenalidomide-mediated IKZF1 protein degradation confers glucocorticoid resistance in RS4;11 cells. **(a)** Lenalidomide-induced IKZF1 protein degradation was validated by Western blot analysis after 24 hours incubation with 1μM or 2μM lenalidomide. GAPDH was used as loading control. (b-c) RS4;11 pre-treated for 24 hours with 1μM lenalidomide were incubated with increasing concentrations of prednisolone (left panel) or dexamethasone (right panel) and relative cell survival was determined by MTS assay **(b)** or AnnexinV/7-AAD staining **(c)**. For MTS assays, all values were normalized to untreated control cells. Error bars represent ± standard error of the mean (SEM) from three independent experiments. For MTS assays, *P* values were calculated based on the differences of the best-fit curve. For AnnexinV/7AAD assays, data represent the means from three independent experiments, and error bars represent SEM. **(d)** Relative expression of glucocorticoid (GC)-responsive target genes *SGK1*, *DFNA5*, *FBXW7*, *PRICKLE1* and *IL1B* in RS4;11 cells displaying loss of IKZF1 function through either lenalidomide-induced IKZF1 protein degradation (upper panel) or *IKZF1*-shRNA expression (lower panel). Expression was measured after 16 hours of 2 μM prednisolone treatment by qRT-PCR and normalized to *HPRT* expression. All expression levels are compared to untreated (UT) parental or NT-shRNA cells. Error bars represent SEM from at least three independent experiments.

IKZF1 alterations mediate glucocorticoid resistance in childhood BCP-ALL patients

To assess whether primary pediatric BCP-ALL patient samples with *IKZF1* alterations displayed GC resistance, we first examined the outcome of MTT assays performed on 187 diagnosis patient samples collected as part of the ALL-9 trial of the Dutch Childhood Oncology Group (DCOG) [31,32]. Data were available for 152 BCP-ALL patients without an *IKZF1* alteration (*IKZF1*-WT) and 35 patients with either an *IKZF1* deletion (*n*=33) or mutation (*n*=2). For each primary leukemic sample, the median lethal concentration (LC_{50}) was determined 96 hours after treatment with one specific type of chemotherapeutic drug, which included prednisolone, dexamethasone, L-asparaginase and vincristine (Supplementary Table 3 and 4). We observed that primary BCP-ALL samples with an *IKZF1* alteration (*IKZF1*-Mut) had a 10-fold higher LC_{50} for prednisolone (P=0.004) and a 20-fold higher LC_{50} for dexamethasone (P=0.0001) relative to *IKZF1*-WT samples (Figure 6). In contrast, LC_{50} values for L-asparaginase and vincristine were not significantly different between the two patient groups. Next, we examined the early *in vivo* day 8 prednisolone response in 646 pediatric patients from DCOG trials ALL-10 and ALL-11, which has been shown to act as an important prognostic parameter in childhood ALL. Strikingly, we found that the frequency of *IKZF1* deletions was significantly higher in patients with a poor prednisolone response compared to good prednisolone responders (15% versus 7%; *P*=0.015; Figure 6). In conclusion, our data demonstrate that pediatric BCP-ALL patients with monoallelic *IKZF1* alterations display resistance to GCs.



Chemotherapeutic drug (MTT assay)	*IKZF1*-WT (N=152) LC_{50} (95% CI)	*IKZF1*-Mut (N=35) LC_{50} (95% CI)	Mann-Witney U (*P*-value)
Prednisolone (μM)	0.88 (0.72-1.14)	9.1 (1.22-77.3)	0.004
Dexamethasone (μM)	0.08 (0.03-0.12)	1.7 (0.21-15.0)	0.0001
L-Asparaginase (U/mL)	0.23 (0.06-0.38)	0.25 (0.05-1.1)	0.292
Vincristine (μM)	0.83 (0.76-3.5)	1.62 (0.76-3.5)	0.290

Day 8 Prednisolone Response (N=646)	*IKZF1*-WT (N=551)	*IKZF1*-Mut (N=95)	Chi-square (*P*-value)
Good responders (92%)	515 (93%)	82 (85%)	0.015
Poor responders (8%)	36 (7%)	13 (15%)	

Figure 6 *IKZF1* gene alterations impact glucocorticoid therapy response in BCP-ALL patients. Primary leukemia samples obtained at the time of diagnosis from pediatric BCP-ALL patients of the Dutch childhood oncology (DCOG) trial ALL-9 were treated with a range of different concentrations of prednisolone (0.02-700 μM), dexamethasone (0.0003-15 μM), L-asparaginase (0.003-10 IU/mL), and vincristine (0.01-60 μM). After 96 hours the samples were measured by MTT assay (right upper table). The median lethal concentration (LC_{50}) and 95% confidence interval (CI) was determined for the patient samples without (*n*=152) or with an *IKZF1* gene deletion or mutation (*n*=35). For statistical analyses of the MTT data the Mann-Whitney U test was performed. In addition, the effect of *IKZF1*-deletion status on the *in vivo* prednisolone response was determined in BCP-ALL patients from the DCOG trials ALL-10 and ALL-11 (right lower panel). The presence (*IKZF1*-Del) or absence of *IKZF1* deletions (*IKZF1*-WT) was determined in prednisolone good responders (*n*=594) and prednsiolone poor responders (n=52). Prednisolone poor responders were defined as patients with ≥ 1000 leukemic blasts/μl blood at day 8 after the initial prednisolone treatment regimen. *P* value was calculated by Chi-square test.

Discussion

In this study, we have identified the tumor suppressor IKZF1 as an important regulator of the apoptotic response to synthetic glucocorticoids (GCs), which are cornerstone drugs in the treatment of lymphoid malignancies. Specifically, we demonstrate that haplodeficiency for *Ikzf1* in normal B cells, silencing of IKZF1 expression in BCP-ALL cell lines, as well as the presence of inactivating *IKZF1* alterations in BCP-ALL lead to GC resistance. In BCP-ALL, *IKZF1* deletions and missense mutations correlate with inferior treatment outcome and are present in about 35% of relapsed ALL cases [10,14,33]. Our findings suggest that leukemic clones harboring *IKZF1* alterations show enhanced survival in response to GC treatment, which likely contributes to an increased risk of relapse.

GC resistance constitutes a serious clinical problem in the treatment of ALL, since relapsed leukemic cells display intrinsic resistance against prednisolone and dexamethasone [18]. Investigation of GC resistance mechanisms has shown that low expression levels and mutations of the NR3C1 gene [34], epigenetic silencing of the pro-apoptotic gene *BIM* [35] , high *CASP1* and *NALP3* expression [36] and increased AKT activation [37] contribute to GC resistance. Other studies have identified novel regulators of GR-dependent gene

transcription that may impact GC therapy responses [38-42]. However, to date only the transcriptional co-factor CBP (encoded by *CREBBP*) and *TBL1XR1* were shown to be genetically altered and enriched in relapsed BCP-ALL [3,7,43], accounting for only a small fraction of the relapsed cases. Thus, in spite of the improved knowledge on the molecular mechanisms through which GCs act, the exact genetic pathways that contribute to GC therapy resistance in the majority of relapsed BCP-ALL cases remain to be elucidated.

Here, we identified IKZF1 as a novel regulator of GC-induced transcriptional responses and a critical determinant of the sensitivity towards GC-mediated cell death in normal and leukemic B cells. *Ikzf1*$^{+/-}$ B cells displayed reduced sensitivity towards GC-induced apoptosis, which correlated with attenuated regulation of GC-responsive target genes. However, it was evident that not all genes were affected in an equal manner and there was a clear difference between individual GR-target genes. Furthermore, all GC-repressed genes examined were already affected in their basal expression levels upon *Ikzf1* haplodeficiency. In addition, reduction of IKZF1 levels in human BCP-ALL cell lines NALM6 and RS4;11 resulted in an attenuated expression of GC-responsive target genes. We propose that IKZF1 affects GR-dependent gene transcription, at least in part through GRE sites, based on the observation that IKZF1 target sites partially overlap with GR binding sites [44]. Whether stimulation of GR-dependent gene transcription by IKZF1 involves recruitment of positive transcriptional co-factors or quenching of negative regulators of GR-dependent gene transcription remains to be determined. Furthermore, we cannot exclude that IKZF1 affects chromatin accessibility of GR in normal and leukemic B cells.

Our studies show that IKZF1 silencing in NALM6 and RS4;11 results in a GC resistance phenotype. Others have demonstrated that *IKZF1* knockdown in the BCP-ALL cell lines REH and UOCB1 had no impact on the sensitivity towards prednisolone-induced apoptosis [45]. However, we found that REH and UOCB1 cell lines are intrinsically resistant towards GCs (Supplementary Figure S9) and are therefore not suitable to study *IKZF1*-mediated GC resistance. Furthermore, our analyses of the BCP-ALL patient samples revealed that not all patient samples with *IKZF1* deletions show a similar protection against GC-induced apoptosis. These findings suggest that either threshold levels of IKZF1 expression, the type of *IKZF1* aberrations, or specific genetic factors may contribute to the resistance phenotype in leukemic cells. Indeed, gene deletions involving *BTG1* and *TBL1XR1*, or inactivating point mutations in *CREBBP* represent candidate genetic factors that could enhance the GC therapy resistance in *IKZF1*-deleted BCP-ALL [3,39], 41. Unfortunately, our cohort of patient samples was too small to analyze the effect of such co-occurring alterations within the *IKZF1*-deleted patient group. Furthermore, other genetic factors may suppress the therapy resistance phenotype, as for instance ERG deletions weaken the prognostic value of *IKZF1* lesions [46,47]. It is also important to note that for leukemia patients receiving chemotherapy treatment, different *in vivo* factors could affect the GC therapy resistance phenotype due to IKZF1 alterations, including the bone marrow microenvironment. Interestingly, consistent with the *in vitro* data of MTT assays on primary BCP-ALL patient samples, analysis of the initial prednisolone response

at day 8 revealed a significant enrichment for *IKZF1* deletions in the poor prednisolone responders. This suggests a novel role for *IKZF1* alterations in predicting GC therapy response by mediating GC-resistance. However, further studies are required to obtain a comprehensive understanding of the complex interplay between loss of IKZF1 function and the specific genetic pathways that modulate GC therapy responses.

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Supplemental Information Chapter 3

Supplemental Tables

Supplementary Table S1 Primer sequences for real-time quantitative PCR and shRNA-vectors

qPCR	Targets	Forward (5'->3')	Reverse (3'->5')
Mouse	Dusp1	TACAAGAGCATCCCTGTGGAGGAC	TGAGGTAAGCAAGGCAGATGGTGG
	Tsc22d3	GGCCCTAGACAACAAGATTG	TCAAGCAGCTCACGAATCTG
	Zfp36l2	ACTCTTGCAATTTCGACCATTACA	CGTCCCTACCGCCTTCTTGTCCAG
	Fkbp5	GCGAAGGAGCACAACGAGCGGGAC	GTTGTTTGCCAGCGGCTCCTTCTA
	Sgk1	ACTGGGATGATCTCATCAATAAGA	TGTCAGGGGACCTGCCGATGGAGC
	Ddit4	ACTGCGAGTCCCTGGACAGCAGCA	GGCTCTCCTGCAGCAGCTGCATCA
	Irs2	AGGCACTGGAGCTTTGCCCTCTGC	AACATGGCGGCGATGGGGCTGGTA
	Il1a	GAGAGGGAGTCAACTCATTGG	AGAGATGGTCAATGGCAGAAC
	Il1b	CACTACAGGCTCCGAGATGAACAA	CTTTGAGGCCCAAGGCCACAGGTA
	Il6	CTAATTCATATCTTCAACCAAGAG	GCCACTCCTTCTGTGACTCCAGCT
	Sdpr	TGCAGTCCATAACCCATGATC	ACAAACTTCTGCCTGACGAG
	Nr3c1	AACTGGAATAGGTGCCAAGG	CCAGGGCTTGAATATCCATT
	Hprt	GGGGGCTATAAGTTCTTTGCTGACC	TCCAACACTTCGAGAGGTCCTTTTCAC
qPCR	**Targets**	**Forward (5'->3')**	**Reverse (3'->5')**
Human	SGK1	ACCTTCTGTGGCACGCCGGA	CTGTGTTTCGGCTATAAAAAGGC
	ZFP36L2	CACACTTCTGTCCGCCTTCTA	GCATGTTGTTCAGGTTGAGGT
	DFNA5	AACTCCAGATCATTCCCACAC	CTGCACAATCCCAAACCTTTC
	FBXW7	GCTACTGGAGAATTTTGGCTG	TGGACTGTGTATGAAACCTGG
	PRICKLE1	ACTGGGAAGAGGAACAATTAAGC	AGACAAAACAGGATGGGTGC
	FKBP5	GGCCATGTGCTACCTGAAGC	ACAAGCCTTTCTCATTGGCACTGTC
	DDIT4	AGGAAGACACGGCTTACCTG	CATCAGGTTGGCACACAAGT
	TSC22D3	CCAGCGTGGTGGCCATAGAC	GGATCTGCTCCTTCAGGATCTCCA
	IL1B	ACTGCACGCTCCGGGACTCACAGC	ACTGCACGCTCCGGGACTCACAGC
	HPRT	GGTCCTTTTCACCAGCAAGCT	TGACACTGGCAAAACAATGCA
shRNA	**Targets**	**sense**	**antisense**
Human	IKZF1	GAAGAATGTGCGGAGGATTT	AAATCCTCCGCACATTCTTC
	Non-targeting (NT)	CGTACGCGGAATACTTCGA	TCGAAGTATTCCGCGTACG

Supplementary Table S2 Ingenuity upstream regulator analyses of microarray expression data mouse B-cells. See online version at Leukemia website.

Supplementary Table S3 Excel data sheet with original MTS data and patient information used in figure 6. See online version at Leukemia website.

Supplementary Table 4 Summary BCP-ALL patient characteristics included in MTT assay.

	Number of samples*	*IKZF1*-WT (N=152)	*IKZF1*-Mut (N=35)	*P*-value Chi-square
Sex				
Male	115 (61%)	92 (61%)	23 (66%)	0.57
Female	72 (39%)	60 (39%)	12 (34%)	
Relapse				
Relapse	56 (30%)	34 (22%)	22 (63%)	<0.001
No Relapse	131(70%)	118 (78%)	13 (37%)	
ETV6-RUNX1				
Translocation	42 (26%)	40 (30%)	2 (6%)	0.007
No translocation	122 (74%)	93 (70%)	29 (94%)	
BCR-ABL1				
Translocation	8 (4%)	2 (1%)	6 (18%)	<0.001
No translocation	170 (96%)	143 (99%)	27 (82%)	
Hyperdiploid				
N>51 chr	42 (25%)	37 (27%)	5 (18%)	0.32
N<51 chr	124 (75%)	101 (73%)	23 (82%)	

Presence or absence of IKZF1 *deletions and mutations (*IKZF1*-Mut) was based on MLPA and targeted sequencing. *Because of missing values, numbers do not always add up to 187 BCP-ALL cases.*

Supplementary Figures

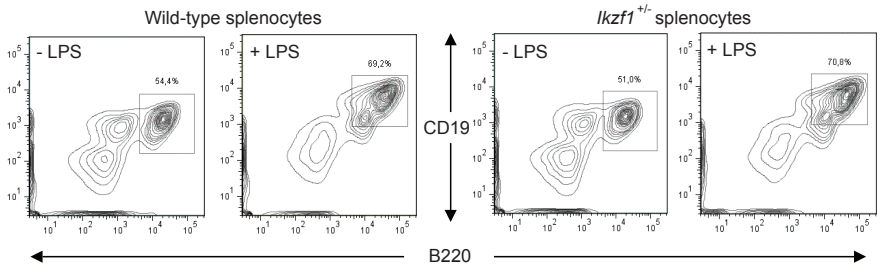


Figure S1 Phenotypic characterization of wild-type and *Ikzf1*$^{+/-}$ splenic B cells. Single cell splenocytes were stimulated with lipopolysaccharide (LPS) for 48 hours and cells were stained before and after LPS treatment with cell surface markers CD19 and B220 and analyzed by flow cytometry. Fraction CD19^{+}B220^{+} cells is not significantly different between wild-type (left two panels) and *Ikzf1*$^{+/-}$ (right two panels) splenic B cells.

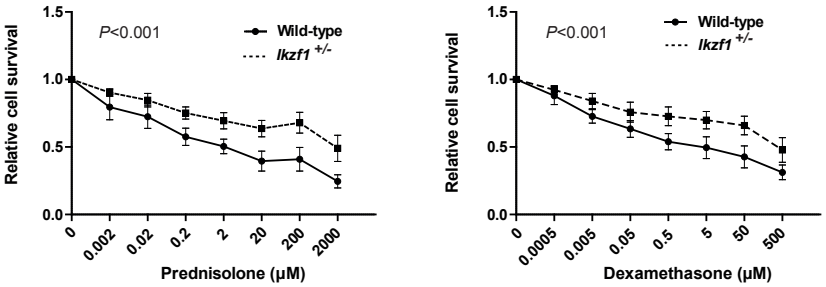


Figure S2 Survival response of CD19⁺ *Ikzf1*$^{+/-}$ progenitor B cells upon glucocorticoid treatment. Wild-type and *Ikzf1*$^{+/-}$ mice (n=6) were treated for 48 hours with increasing concentrations of prednisolone (left panel) or dexamethasone (right panel) and analyzed by MTS assay. All values were normalized to untreated B cells. Error bars represent ± standard error of the mean (SEM). P values (two-sided ANOVA) were calculated based on the differences of the best-fit curve and are shown in each panel.

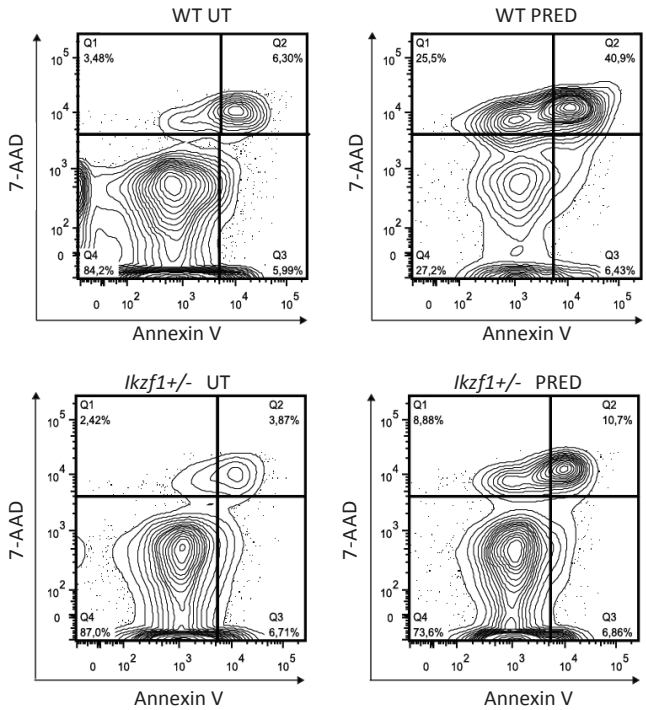


Figure S3 AnnexinV staining of *Ikzf1*$^{+/-}$ B cells treated with glucocorticoids. Wild-type (WT) and *Ikzf1*$^{+/-}$ splenic B cells pre-treated by lipopolysaccharide (LPS) were cultured for 48 hours with 2μM prednisolone and analyzed by AnnexinV (PE-conjugated)/ 7-AAD staining. Plots show representative data from FACS analyses measuring 10.000 events, excluding cellular debris, from samples untreated (UT) or treated with 2μM prednisolone (PRED) for 48hours.

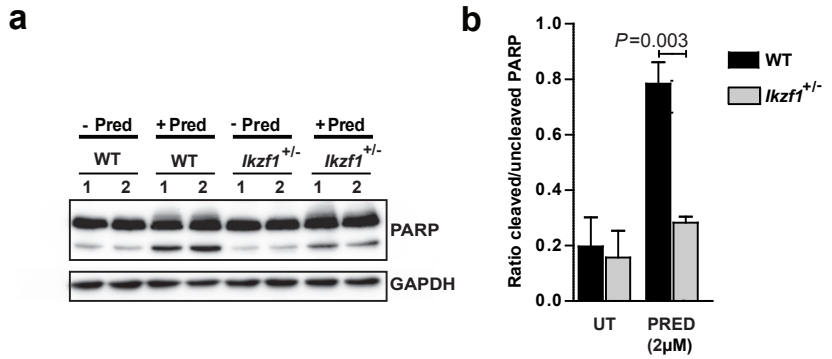


Figure S4 Analysis of glucocorticoid resistance phenotype in *Ikzf1*$^{+/-}$ B cells by PARP cleavage. **(a)** Western blot analysis to detect protein levels of cleaved PARP as marker for apoptosis in wild-type (WT) and *Ikzf1*$^{+/-}$ B cells after 16 hours treatment with 2μM prednisolone. GAPDH was used as loading control. **(b)** Quantification of cleaved versus uncleaved PARP ratio for WT and *Ikzf1*$^{+/-}$ B cells after 16 hours treatment with 2 μM prednisolone. Data are means, and error bars represent SEM of three independent experiments.

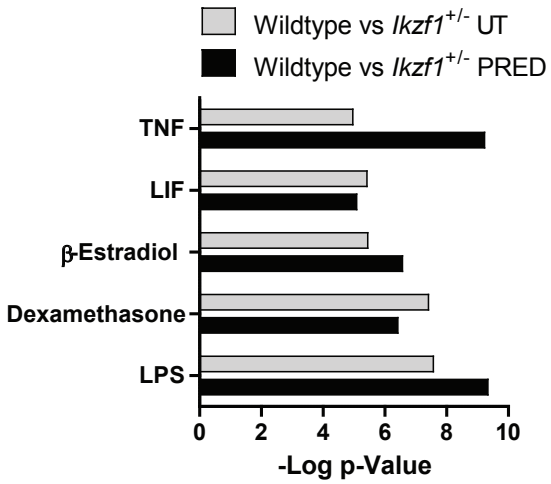
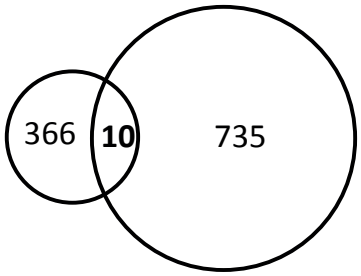


Figure S5 Identification of glucocorticoid-responsive target genes in *Ikzf1*$^{+/-}$B cells. Microarray expression analyses was performed on RNA isolated from wild type (WT) and Ikzf1+/- splenic B cells in the absence (untreated, UT) or presence of a 16 hours prednisolone treatment. Ingenuity pathway analyses on differentially expressed genes between wild-type (WT) and *Ikzf1*$^{+/-}$ B-lymphocytes identified upstream regulatory pathways that were commonly affected in both control and prednisolone treated samples.

a

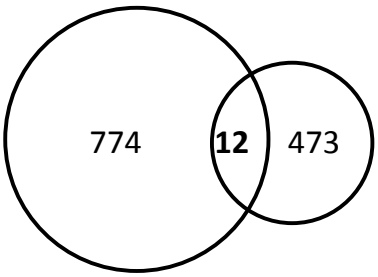
Up-regulated Genes in Ikzf1$^{+/-}$ vs WT mice
VS. *IKZF1*-deleted vs. WT patients



10 Overlapping genes
DENND1A
EVI2A
GNA15
IFITM3
INPP1
OLFML2A
PSTPIP2
SLC12A9
SPSB2
TMEM141

b

Down-regulated Genes in Ikz1$^{+/-}$ vs WT mice
VS. *IKZF1*-deleted vs. WT patients



12 Overlapping genes
BAZ1B
BCL9
CCNG2
CYP46A1
EPOR
ITGB1BP2
LONRF1
MCM9
PHF8
PKIA
PLCB4
PLEKHH2

Figure S6 Comparison gene expression signature between mouse B cells and human leukemic blasts displaying loss of IKZF1 function. **(a)** Schematic representation of the overlap between genes that were differentially up-regulated in the mouse B cell dataset comparing wild-type with *Ikzf1*$^{+/-}$ B cells (Marke et al; this manuscript), and genes that were up-regulated in the human dataset comparing BCP-ALL blasts of patients without or with and *IKZF1* deletion (Vitanza et al; 2014). **(b)** Schematic representation of the overlap between genes that were differentially down-regulated in the mouse B cell dataset comparing wild-type with *Ikzf1*$^{+/-}$ B cells (Marke et al; this manuscript), and genes that were down-regulated in the human dataset comparing BCP-ALL blasts of patients without or with and *IKZF1* deletion (Vitanza et al; 2014).

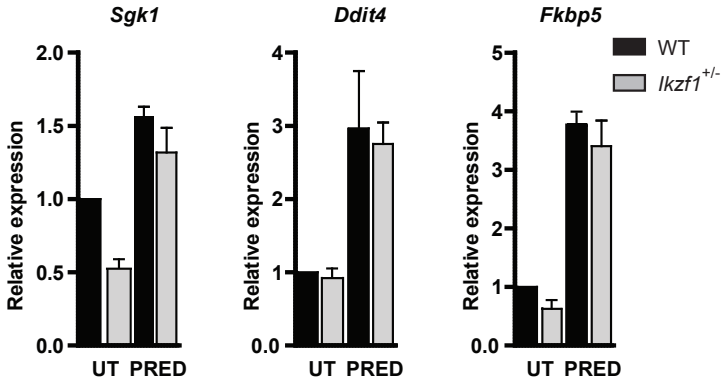


Figure S7 Expression levels of glucocorticoid-responsive target genes in mouse B cells not affected by Ikzf1-haplodeficiency. B cells pretreated with lipopolysaccharide (LPS) were cultured for 16 hours with 2μM prednisolone and subsequently RNA was isolated. Transcript levels of glucocorticoid (GC)-induced target genes *Sgk1*, *Ddit4* and *Fkbp5* were measured by quantitative reverse transcription polymerase chain reaction in B cells from wild-type (WT) and *Ikzf1*$^{+/-}$ mice. All expression levels were normalized to *Hprt* expression and compared to the levels in the untreated (control) WT B cells. Data are means, and error bars represent SEM from four independent experiments performed in duplicate.

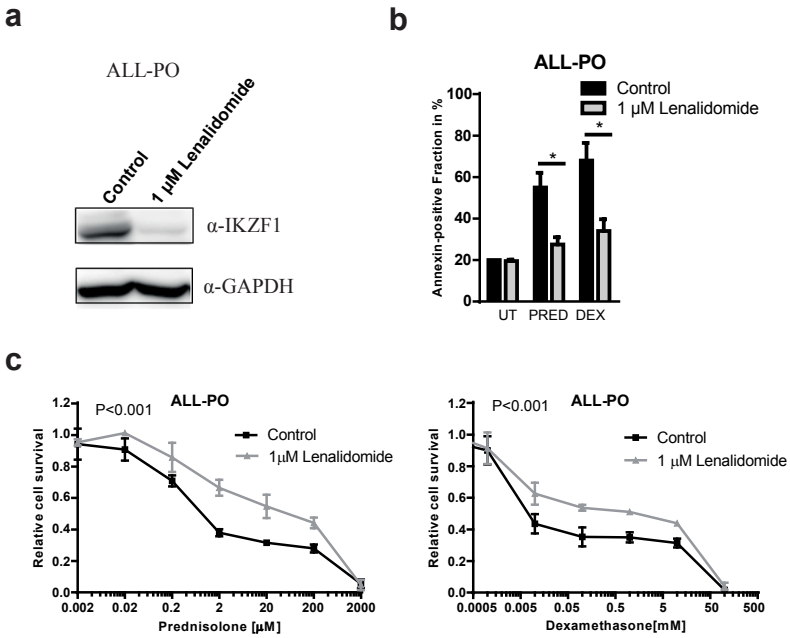


Figure S8 Silencing of IKZF1 by lenalidomide inhibits glucocorticoid-induced apoptosis in ALL-PO cell line. **(a)** Effect of lenalidomide treatment on IKZF1 protein levels in ALL-PO cells. GAPDH was used as loading control. **(b)** AnnexinV/7-AAD staining on ALL-PO cells that were pretreated for 16 hours with or without lenalidomide (control) and subsequently incubated for 48 hours with either prednisolone (PRED) or dexamethasone (DEX) and compared to untreated (UT) cells. **(c)** Relative cell survival of ALL-PO cells pretreated for 16 hours with lenalidomide and incubated with increasing concentrations prednisolone and dexamethasone.

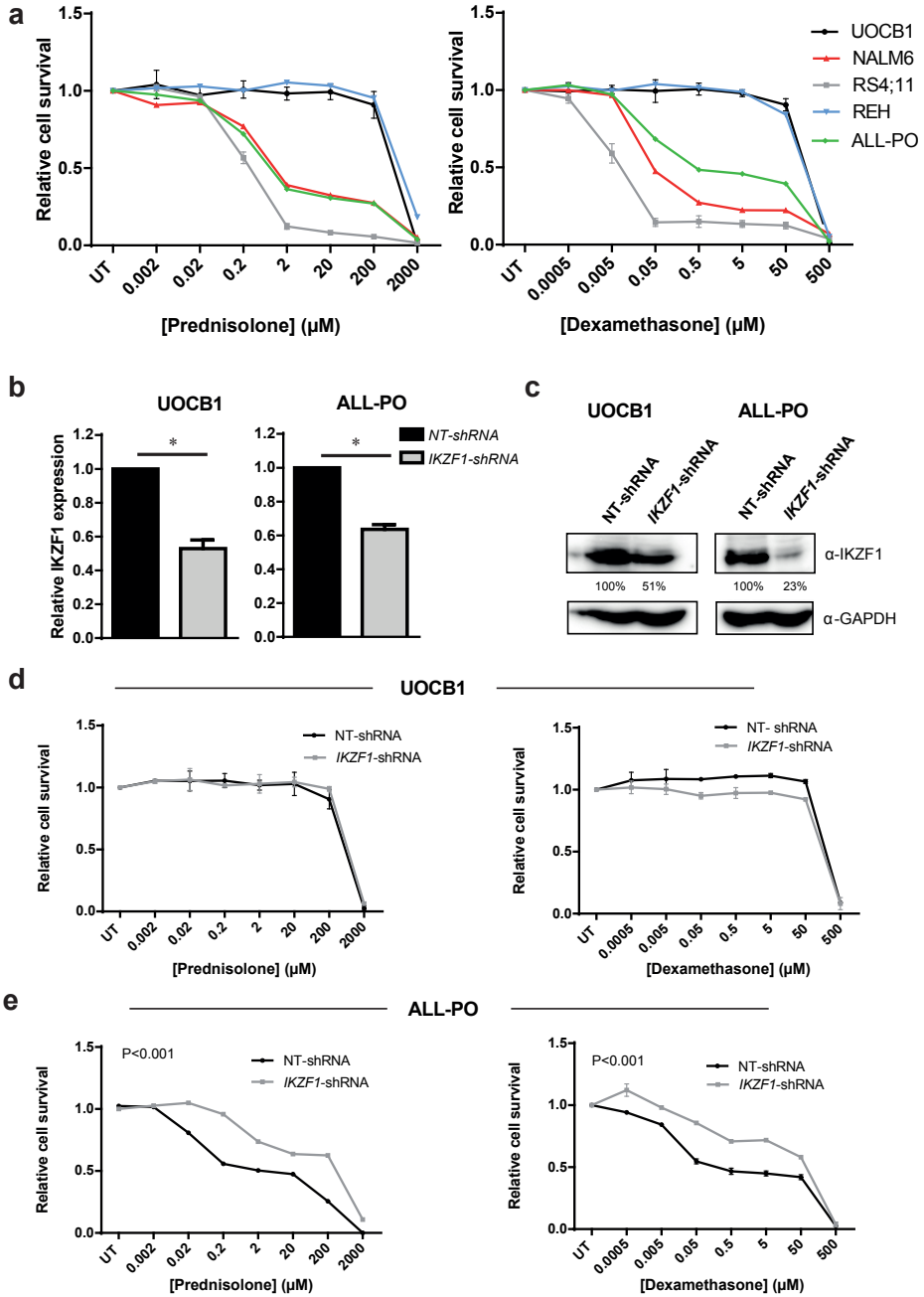

a
Relative cell survival
1.5
1.0
0.5
0.0
UT
0.002
0.02
0.2
2
20
200
2000
[Prednisolone] (μM)
UT
0.0005
0.005
0.05
0.5
5
50
500
[Dexamethasone] (μM)
UOCB1
NALM6
RS4;11
REH
ALL-PO
b
UOCB1
ALL-PO
Relative IKZF1 expression
*
NT-shRNA
IKZF1-shRNA
c
UOCB1
ALL-PO
NT-shRNA
IKZF1-shRNA
α-IKZF1
100%
51%
100%
23%
α-GAPDH
d
UOCB1
NT-shRNA
IKZF1-shRNA
NT- shRNA
e
ALL-PO
P<0.001

Figure S9 Effect of IKZF1 knockdown on glucocorticoid-resistant and glucocorticoid-sensitive BCP-ALL cell lines. **(a)** Relative cell survival was determined in the BCP-ALL cell lines UOCB1, REH, RS4;11, NALM6 and ALL-PO after 48 hours treatment with increasing concentrations of prednisolone or dexamethasone using MTS assay. **(b-c)** Efficiency of IKZF1 knockdown using lentiviral expression of non-targeting (NT) shRNA or *IKZF1*-shRNA in cell lines UOCB1 and ALL-PO, as determined by quantitative reverse transcription PCR (b) or Western blot analysis using GAPDH as loading control (c). **(d-e)** Relative cell survival of UOCB1 (d) and ALL-PO (e) cell lines expressing either NT-shRNA or *IKZF1*-shRNA in response to increasing concentrations prednisolone or dexamethasone.

Chapter 4

PTEN/AKT Pathway drives Glucocorticoid Resistance in *IKZF1*-deleted B Cell Precursor Acute Lymphoblastic Leukemia

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Manuscript in preparation

Abstract

Resistance to glucocorticoid therapy remains an important clinical problem in the treatment of B cell precursor acute lymphoblastic leukemia (BCP-ALL). We have recently shown that loss of IKZF1 function promotes resistance to glucocorticoid-induced apoptosis, compromising the therapeutic efficacy of glucocorticoids (GCs). In T-ALL, inactivation of PTEN has been linked to glucocorticoid resistance, but in BCP-ALL such a role for PTEN has not been reported. Here, we identify the PTEN/AKT axis as the main driver of GC resistance in *IKZF1*-deleted leukemic cells (*IKZF1*^{KO}). Loss of *IKZF1* resulted in decreased PTEN mRNA and protein expression in mouse B cells as well as in BCP-ALL leukemic cell lines and involves upregulation of the transcription factor HES1. Silencing of *PTEN* expression in Nalm6 pre-B ALL cells phenocopied the GC resistance phenotype observed in *IKZF1*^{KO} cells. Targeted deletion of *IKZF1*, or shRNA-mediated knockdown of *PTEN*, resulted in activation of the AKT pathway, and increased AKT-dependent phosphorylation of the glucocorticoid receptor (GR). Moreover, pharmacological inhibition of AKT re-sensitized *IKZF1*^{KO} cells to GCs and restored GC-target gene regulation, both in BCP-ALL cell line models and in patient-derived xenografts. Collectively, our data show that there are unexpected parallels between mechanisms driving GC therapy resistance in T- versus BCP-ALL and that therapeutic targeting of AKT in *IKZF1*-deleted ALL can restore response to GCs.

Introduction

During the initial treatment of patients with B cell precursor acute lymphoblastic leukemia (BCP-ALL), the early response to glucocorticoids (GCs) single-agent treatment is a strong predictor of outcome [1]. In addition, challenging leukemic blasts *ex vivo* with GCs can be used to predict therapy response *in vivo* [2,3]. Therefore, resistance to GCs can be considered as an adverse prognostic factor in BCP-ALL [4]. Indeed, GC resistance remains a significant problem in the treatment of BCP-ALL, since relapsed leukemic cells display intrinsic resistance towards prednisolone and dexamethasone [3]. Studies on mechanisms of GC resistance in ALL have shown that low expression levels and mutations of the glucocorticoid receptor (GR) [5], epigenetic silencing of the pro-apoptotic gene *BIM* [6], high *CASP1* and *NALP3* expression [7] and increased AKT activation [8] contribute to GC resistance. Other studies have identified novel regulators of GR-dependent gene transcription that may impact GC therapy responses [9-13]. Our lab has recently shown that loss of tumor suppressor *IKZF1* confers resistance to GCs in both mouse and human model systems as well as primary BCP-ALL patient samples [14]. *IKZF1* aberrations are frequently observed in BCP-ALL and represent an independent poor prognostic factor in BCP-ALL [15-18]. However, the exact molecular pathways that contribute to GC therapy resistance *IKZF1*-deleted BCP-ALL cases have not yet been resolved. The tumor suppressor PTEN is the main negative regulator of the PI3K/AKT pathway and PTEN disfunction is a common event in approximately 10% of all pediatric T-ALLs [19]. *NOTCH1* activating mutations, which are common events in T-ALL [19], can downregulate *PTEN* via the transcription factor HES1 [20]. Loss of PTEN function leads to hyperactivation of the AKT pathway, which has been linked to GC resistance in T-ALL [8]. However, much less is known about the role of PTEN in BCP-ALL, as PTEN mutations are not observed in BCP-ALL.

Here we show that GC therapy resistance in *IKZF1*-deleted ALL leads to loss of PTEN expression, which appears to involve upregulation of the transcription factor HES1. Of note, HES1 was previously identified as a target of IKZF1-mediated transcriptional repression in erythroid and T cells [21,22] Furthermore, we show that IKZF1-mediated GC resistance can be effectively reversed by inhibition of AKT both in cellline models and primary patient samples.

Methods

Primary patient samples

Bone marrow patient samples were obtained from the Dutch Childhood Oncology group (DCOG). In accordance with the Declaration of Helsinki, written informed consent was obtained from parents or legal guardians, and institutional review boards approved the use of excess diagnostic material for research purposes.

Cell culture

HEK293-FT cells and BCP-ALL cell lines, RS4;11 and Nalm6, were cultured in DMEM GlutaMAX medium (Life Technologies) or RPMI 1640 GlutaMAX medium (Life Technologies), respectively. Culture media were supplemented with 10% heat inactivated fetal calf serum (HI-FCS) and 1% Penicillin/Streptomycin (P/S) (Invitrogen). Cells were maintained in a humidified incubator at 37 °C with 5% CO2.

Lentiviral production and transduction

Viral particles were produced by transient transfection of HEK293-FT cells with a combination of pLKO.1, ps-PAX2 and VSV-G using Lipofectamine 2000 (Invitrogen. After 48 hours, viral supernatant was harvested, filtered, concentrated and leukemia cell lines were transduced in the presence of 1µg/mL polybrene via spin inoculation and 24 hours after transduction selected with 1µg/mL puromycin. The guide RNA (gRNA) for *IKZF1* in exon 3 was cloned into the lentiviral vector pLKO.1 containing CAS9. CRISPR/Cas9-mediated genome editing was confirmed via Surveyor® Mutation Detection in cell pools. Subsequently, *IKZF1*-gRNA and NT-gRNA transduced cells were subcloned and screened for IKZF1 expression via western blotting and sequencing. For shRNA experiments, cells were generated as described previously [14].

Cell viability assays

To measure therapy response in the model cell lines, a number of different cell viability assays was used. BCP-ALL cell lines were cultured for 48 hours in the absence or presence of the chemotherapeutic agents. Amine staining with the LIVE/DEAD™ Fixable Dead Cell Stain Sampler Kit (Thermo-Fischer, L34960) was performed according to the manufacturers protocol (For representative analysis, see supplemental Figure 1). For MTS assays, cells were seeded at a density of $1.0*10^5$ in a flat bottom 96-wells plate. Cell were treated with a broad variety of single agent chemotherapies in an increasing concentration and incubated for 72 hours. After incubation, 20 µL Cell Titer Aqueous ONE Solution (Promega) was added to each well and incubated for 3 hours and cell viability was determined via measuring the absorbance at 492 nm. Absorbance data was normalized to the untreated sample for each cell type.

Mouse experiments and generation of patient-derived xenografts

Generation of C57BL/6J *Ikzf1*$^{+/-}$ mice and experimental procedure to obtain B cells has been previously described [14,23]. For patient-derived xenograft (PDX) models, NOD.*Cg-Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ mice (NSG-mice) were used. Mice were maintained under specific pathogen-free conditions at our Central Animal Laboratory facility. All animal experiments were approved by the Animal Experimental Committee of the Radboud university medical center and were performed in accordance with institutional and national

guidelines. For the expansion of primary leukemic cells, non-conditioned NSG mice were injected intrafemurally with 0.5-1x10^6 viable cells. After 2-4 months, most of these mice developed leukemia with an immunophenotype and genetic composition similar to the original sample at the time of injection. Leukemic cells were harvested by flushing femurs with RPMI medium (Gibco//Life technologies Europe BV, Bleiswijk, The Netherlands) containing 10% FBS (Greiner Bio-One BV, Alphen a/d Rijn, The Netherlands) and 50μM β-mercaptoethanol (Gibco). Leukemic blasts from spleen were isolated through a 70μM filter using RPMI medium containing 10% FBS and 50μM β-mercaptoethanol. Leukemic cells were cryopreserved as viable cell suspension in FBS supplemented with 10% DMSO.

Quantitative RT-PCR

RNA was extracted using a RNeasy minikit (Qiagen) according to the manufacturer´s protocol. This was followed by Superscript cDNA synthesis (*Bio-Rad*, *Hercules*, *CA*), according to manufacturer´s instructions. mRNA expression levels in NALM6, RS4;11 and murine B cells were determined by quantitative PCR reactions using *Power SYBR® Green PCR master mix* (Applied Biosystems, Carlsbad, CA) in combination with mouse specific or human specific primers in the CFX96 TouchTM Real-Time PCR detection system (*Bio-Rad*, *Hercules*, *CA*, *USA*). Mouse and human mRNA expression levels were normalized to mouse and human HPRT respectively. *HPRT* mRNA expression was used as a reference to obtain the relative fold expression of target genes using the comparative cycle threshold $2^{(-\Delta\Delta Ct)}$ method. A complete list of primers can be found in Supplemental Table 1.

Western blot analysis

Leukemic cells as well as murine splenocytes were lysed in 2xLaemmli protein sample buffer and treated with benzonuclease for 30 minutes, prior to boiling. Protein lysates were separated by SDS-PAGE and transferred to PVDF membranes (Amersham Biosciences). After protein transfer, membranes were blocked according to the specific antibody and stained with primary antibodies (See Supplemental Table 2), washed in PBS-0.02% Tween, followed by a horseradish peroxidase (HRP) conjugated secondary antibody. Proteins were visualized with ECL reagent (GE Healthcare) and expression was detected with Fluorchem (Cell biosciences, Santa Clara, USA). Quantification was performed with AlphaView software version 3.3.10 (Cell biosciences, Santa Clara, USA). For cellular fractioning, LPS-activated B cells isolated from wild-type and *Ikzf1*$^{+/-}$ mice were incubated in the presence of 1μM prednisolone for 4 hours. Subsequently, cytoplasmic and nuclear extracts were prepared using a nuclear extraction kit (Abcam, ab113474) according to the manufacturer's protocol.

Ex Vivo Co-culture of Patient-derived Xenograft Leukemic Cells

The ex vivo co-culture model has been described previously [24]. In short, hTERT immortalized human mesenchymal stromal cells (MSCs) [25,26] were seeded in a 96-wells

format and allowed to settle for 24 hours prior to the addition of ALL xenografts. ALL cells were allowed to settle for 24 hours before prednisolone with or without MK2206 was added in increasing concentrations. After 3 days of incubation, cell death was analyzed by Amine staining using flow cytometry (for details see Flow Cytometry).

AKT kinase Inhibition

Cells were treated with prednisolone or dexamethasone in continuous presence of 0.5 μM MK2206 and analyzed by MTS and Amine assays (48h) or used for RNA isolation (16h).

Flow Cytometry

Leukemic cells were analyzed by Fluorescence Activated Cell Sorting (FACS) using an LSRII flow cytometer (BD Biosciences, Breda, The Netherlands). Cells were stained with LIVE/DEAD™ Fixable Dead Cell Stain Sampler Kit (Thermo-Fischer, L34960) according to manufacturer's instructions. For co-culture experiments, MSCs were gated out via FSC/SSC gating. A representative gating strategy can be found in supplemental Figure 2. The data were collected and analyzed by FlowJo V10 software (FlowJo, Ashland, Oregon).

Statistical analyses

Statistical analyses for the cell viability assays and quantitative real-time polymerase chain reaction (qRT-PCR) were performed using PRISM6 (GraphPad Software, La Jolla, CA), and for the MTT data of patient samples, the SPSS program (IBM, Amsterdam, Netherlands) was used. For MTS assays, two-sided ANOVA was performed to assess differences between the best-fit curves. For the Amine staining and qRT-PCR, student's t tests were performed. For the patient data (MTT assay), the statistical significance in the *IKZF1*-deleted group versus *IKZF1*-WT group was calculated using the best fit under the curve method. All statistical tests were two-sided and P values < 0.05 were considered statistically significant.

Results

IKZF1 regulates PTEN expression and AKT signaling in normal and leukemic B cells

We and others have shown that *IKZF1* alterations confer therapy resistance to leukemic cells via distinct mechanisms, including enhanced cell adhesion and modulation of the glucocorticoid response [14,27]. However, the molecular mechanisms underlying these effects are poorly understood. Here, we investigated whether *IKZF1*-mediated GC resistance in BCP-ALL may involve deregulation of the PTEN/AKT route.

IKZF1-mediated regulation of the PTEN/AKT pathway was investigated in BCP-ALL cell line Nalm6, where IKZF1 expression was knocked out by CRISPR/CAS9-medated gene targeting of *IKZF1* Exon 3. Subsequently, *IKZF1*-gRNA (*IKZF1*^{KO}) and non-targeting gRNA (*IKZF1* NT Control) transduced cells were subcloned and screened for IKZF1 expression by western blotting. Indeed, *IKZF1*^{KO} cells showed a significant reduction in IKZF1 protein levels, which was accompanied by diminished expression of PTEN both at the mRNA and protein level (Figure 1A-B). Consistent with a loss in PTEN expression, we observed activation of AKT and increased levels of GR-Ser134 phosphorylation as compared NT-control cells (Figure 1B). In T-ALL, activation of AKT leads to hyperphosphorylation of the GR on serine 134, which leads to suppression of GR function and GC resistance [8]. Increased phosphorylation of the GR, as observed in the *IKZF1*^{KO} cells was associated with a diminished response to glucocorticoids (Figure 1C). The same effects, i.e. loss of PTEN expression, increased phosphorylation of AKT and GC resistance, were seen in response to short hairpin RNA (shRNA)-mediated *IKZF1* gene knockdown in Nalm6 and RS4;11 cells, which we generated previously [14] (Supplemental Figure 3A-B).

To demonstrate that the effects on PTEN expression were a direct consequence of *IKZF1* loss, and independent of leukemic context, we generated LPS-activated B cells from *Ikzf1*$^{+/-}$ mice. We have shown earlier that these cells are about 100-fold more resistant to synthetic GCs, relative to B cells derived from *Ikzf1* wild-type mice [14]. Indeed, similar to Nalm6 cells, Pten mRNA and protein expression levels were strongly reduced in *Ikzf1*$^{+/-}$ B cells compared to wild-type (wt) control B cells (Figure 2 A-B). Consistent with the notion that PTEN is a negative regulator of the AKT pathway, *Ikzf1*-haploinsufficiency led to increased levels of AKT phosphorylation on Ser473 (Figure 2B). Western blot analysis revealed that, similar to T-ALL cases, AKT-mediated phosphorylation of GR was strongly increased in *Ikzf1*$^{+/-}$ B cells (Figure 2B). In T-ALL, phosphorylation of GR on Ser134 appears to interfere with its nuclear translocation [8, 28]. Similarly, we observed that nuclear translocation after GC treatment was significantly impaired in *Ikzf1*$^{+/-}$ cells relative to wild-type cells (Figure 2C). We conclude from these experiments that loss of IKZF1 is associated with diminished PTEN expression, leading to activation of AKT and subsequent hyperphosphorylation of the GR on Serine 134 both in healthy and leukemic B cells.

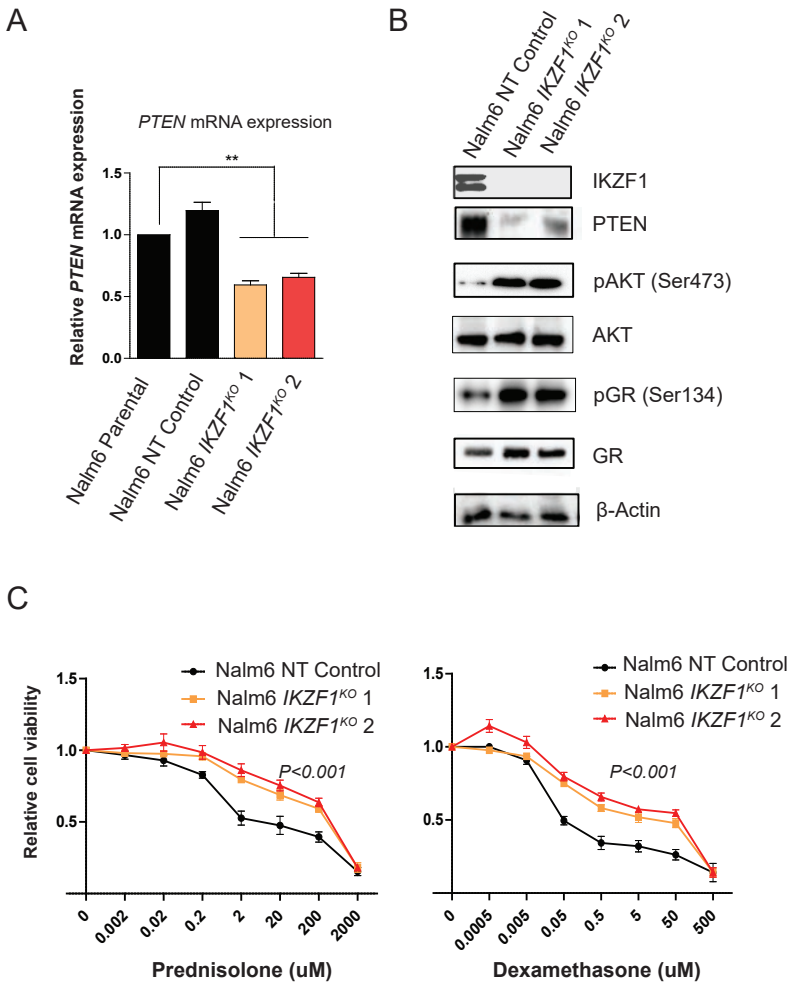


Figure 1 IKZF1 silencing reduces PTEN expression and activates AKT pathway in NALM6 cells. IKZF1 expression was silenced in Nalm6 cells by CRISPR/CAS9-mediated genome editing and two independent subclones of *IKZF1*^{KO} cells (*IKZF1*^{KO} 1 and *IKZF1*^{KO} l 2) were compared to non-targeting-gRNA (NT Control). **(A)** mRNA transcript levels of PTEN were measured in Nalm6 *IKZF1*^{KO} cells in comparison to non-targeting controls by quantitative reverse transcription PCR (qRT-PCR) and normalized to Hprt expression. (B) IKZF1, PTEN, AKT, pAKT(Ser473), pGR(Ser134) and GR protein expression levels of Nalm6 *IKZF1*^{KO} cell lysates were analyzed by western blot. Non-targeting controls were used as control and actin was used as a loading control **(B)** NALM6 NT controls or *IKZF1*^{KO} cells were treated for 48 hours with increasing concentrations of prednisolone or dexamethasone and analyzed using an MTT based viability assay. All values were normalized to untreated Nalm6 cells. Error bars represent ± standard error of the mean (SEM). **(C)** Data represent means, and error bars represent SEM. *P* values (two-sided t test) are indicated. **P*<0.05, ***P*<0.01, and ****P*<0.001.

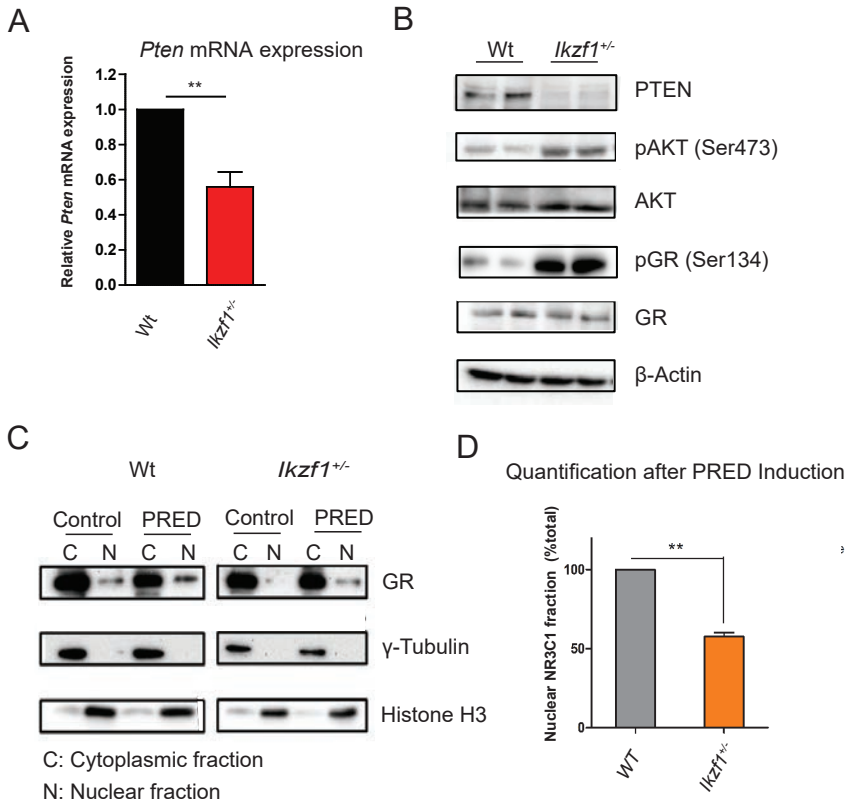


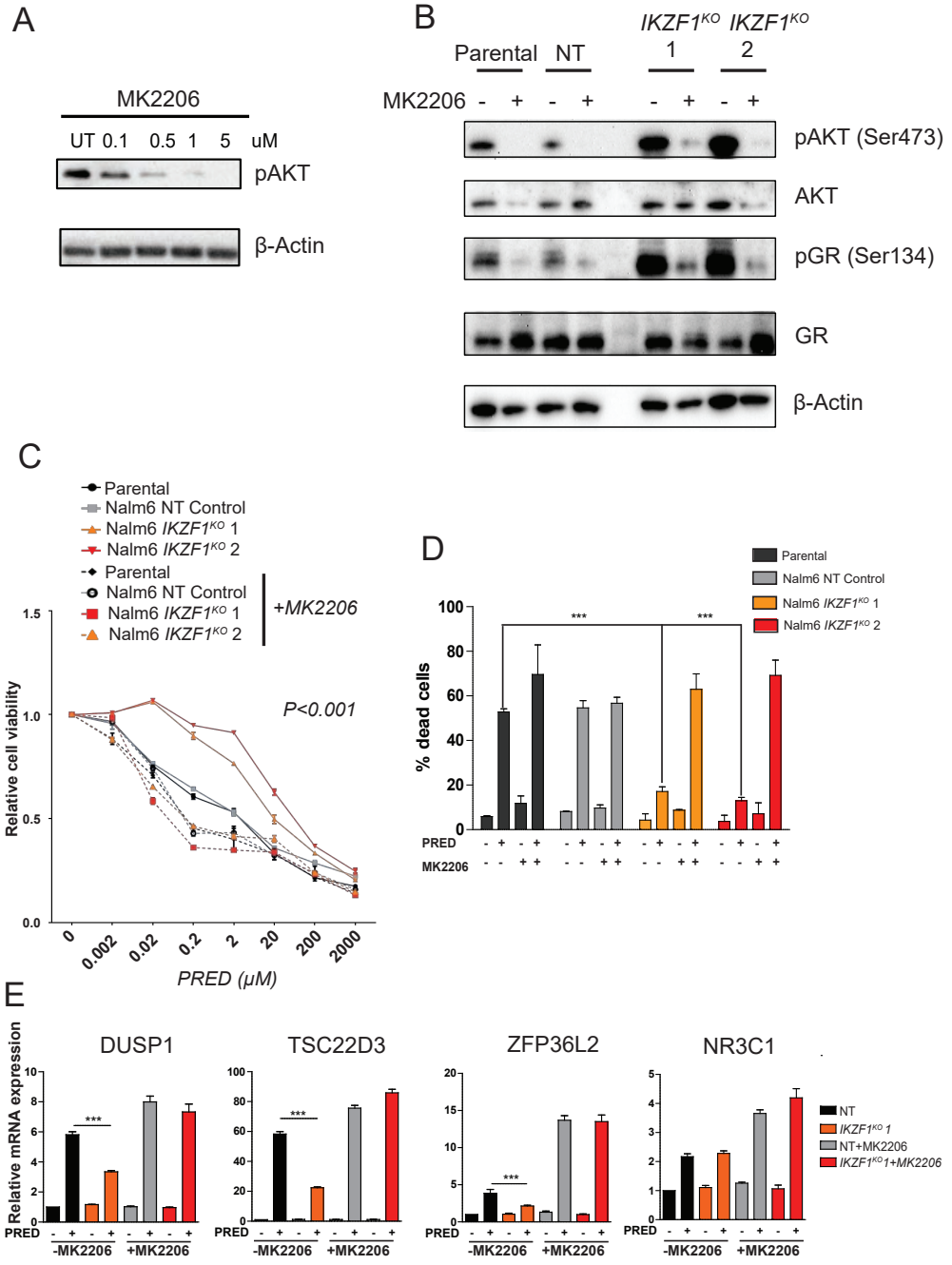
Figure 2 *Ikzf1*-haploinsufficiency downregulates PTEN expression and activates AKT signaling in B cells. (A) mRNA transcript levels of PTEN were measured in LPS-activated B cells from wild-type and *Ikzf1*$^{+/-}$ mice by quantitative reverse transcription PCR (qRT-PCR) and normalized to Hprt expression. **(B)** PTEN, AKT, p-AKT, p-GR and GR protein expression levels of LPS-activated B cells obtained from 4 mice (2 wild-type versus 2 *Ikzf1*$^{+/-}$ mice) were analyzed by western blot analysis. Actin was used a loading control **(C)** Cellular localization analysis of GR via nuclear and cytoplasmic cell fractionation in LPS-activated B cells from wild-type versus *Ikzf1*$^{+/-}$ mice. Tubulin was used as loading control for the cytoplasmic fraction, whereas Histone H3 functions as nuclear loading control. **(D)** Quantification of the relative nuclear GR fraction in wild-type versus *Ikzf1*$^{+/-}$ murine splenocytes after 4-hour induction with 2μM prednisolone. Data represent means, and error bars represent SEM. *P* values (two-sided t test) are indicated. **P*<0.05, ***P*<0.01, and ****P*<0.001.

Inhibition of AKT signaling restores GC-sensitivity in *IKZF1*^{KO} cells

Hyperphosphorylation of AKT leads to GC resistance in T-ALL [8]. To test whether AKT functions as a mediator of GC resistance in *IKZF1*-deleted BCP-ALL cell lines as well, the pharmacological AKT inhibitor MK2206 was used. Western blot analysis showed that incubation with 0.5μM MK2206 reduced phospho-AKT (Ser473) and phospho-GR (Ser134) levels in Nalm6 *IKZF1*^{KO} cells to about 5-10%, and effectively interfered with AKT activation in IKZF1KO cells. (Figure 3B). Moreover, MK2206 treatment of *IKZF1*^{KO} cells restored GC sensitivity to levels observed in *IKZF1*^{WT} cells. (Figure 3C-3D). The effects of MK2206 on reversing GC therapy resistance were further confirmed in Nalm6 and RS4;11 transduced with *IKZF1*-shRNA (Supplemental Figure 3C).

To further explore the effects of AKT inhibition on GC-dependent target gene regulation, quantitative reverse transcription PCR (qRT-PCR) was performed for GR target genes *DUSP1*, *TSC22D3* and *ZFP36L2*, which we have shown previously to be attenuated by loss of IKZF1 [14]. Incubation of control cells with MK2206 enhanced activation of all three GC-target genes in Nalm6. Moreover, we observed that the attenuated response of GC target genes in Nalm6 *IKZF1*^{KO} cells was restored by AKT inhibition (Figure 3F). Importantly, *NR3C1* mRNA levels were not affected by loss of IKZF1, showing that the effects of *IKZF1* on GR mediated gene expression involve regulation of GR activity rather than regulation of its expression.

Figure 3 Inhibition of AKT restores GC-resistance in *IKZF1*^{KO} cells (A) p-AKT protein levels after incubation with increasing concentrations of M2206 for 24 hours analyzed by western blot. Actin was used as a loading control. **(B)** AKT, p-AKT, p-GR and GR protein expression levels of Nalm6 *IKZF1*^{KO} cell lysates after incubation with or without 0.5μM MK2206 for 24 hours were analyzed by western blot. As controls, parental NALM6 and non-targeting Nalm6 were used as control. Actin was used as loading control. **(C)** Nalm6 parental, non-targeting controls or *IKZF1*^{KO} cells were treated for 48 hours with increasing concentrations of prednisolone with or without 0.5μM MK2206 and analyzed using an MTT based viability assay. All values were normalized to untreated Nalm6 cells. Error bars represent ± standard error of the mean (SEM). **(D)** Quantification of Amine stainings measured in Nalm6 *IKZF1*^{KO} cells in comparison to non-targeting controls treated with 2μM Prednisolone with or without 0.5uM MK2206. All experiments were repeated three times. **(E)** mRNA transcript levels of GC-target genes were measured in Nalm6 *IKZF1*^{KO} cells in comparison to non-targeting controls after 24-hour incubation with 2μM Prednisolone with or without 0.5μM MK2206 by quantitative reverse transcription PCR (qRT-PCR) and normalized to *Hprt* expression. Data represent means, and error bars represent SEM. *P* values (two-sided t test) are indicated. **P*<0.05, ***P*<0.01, and ****P*<0.001.


A
MK2206
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pAKT
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B
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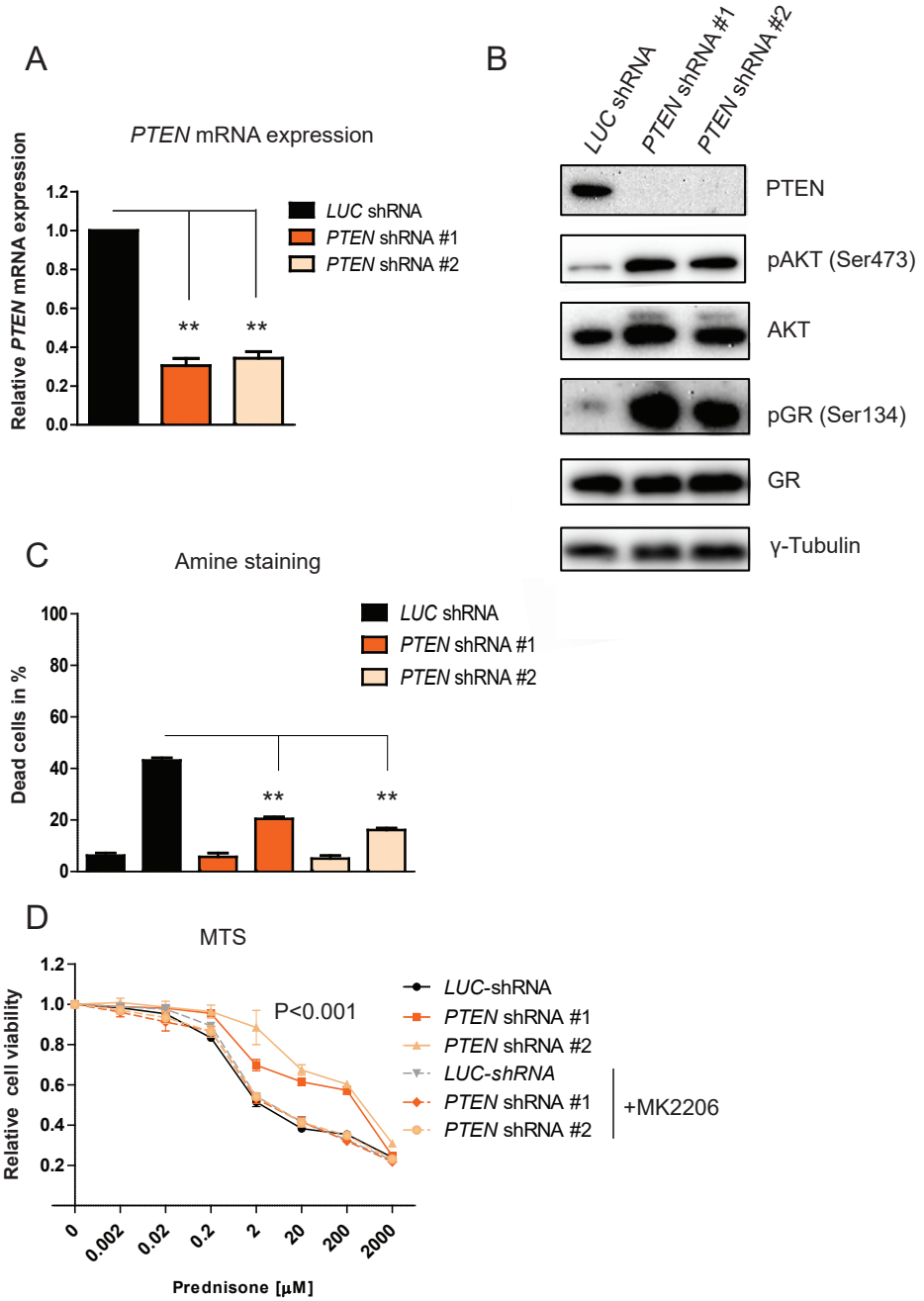
MK2206 - + - + - + - +
pAKT (Ser473)
AKT
pGR (Ser134)
GR
β-Actin
C
Parental
Nalm6 NT Control
Nalm6 IKZF1KO 1
Nalm6 IKZF1KO 2
+MK2206
P<0.001
Relative cell viability
PRED (μM)
D
% dead cells
PRED
MK2206
E
DUSP1
TSC22D3
ZFP36L2
NR3C1
Relative mRNA expression
-MK2206
+MK2206
NT
IKZF1KO 1
NT+MK2206
IKZF1KO1+MK2206

Silencing of *PTEN* induces GC resistance in BCP-ALL

To confirm the pivotal role of PTEN in mediating GC resistance in BCP-ALL, we performed shRNA-mediated knockdown of *PTEN* in BCP-ALL cell line Nalm6. Lentiviral transduction with two independent *PTEN*-shRNAs resulted in a significant decreased PTEN expression, both at RNA (Figure 4A) and protein (Figure 4B) level. Western blot analysis revealed that knockdown of PTEN led to a significant increase in phospho-AKT (Ser473) levels and subsequent phosphorylation of GR on the AKT-specific substrate site Ser134, as compared to the control lysates (Figure 4B). In addition, both *PTEN*-shRNAs conferred resistance to prednisolone in comparison to non-targeting controls as measured by amine staining (Figure 4C) and by by MTS assays (Figure 4D). In addition, treatment of *PTEN*-knockdown cells with MK2206 restored the sensitivity to GCs, indicating that the observed GC resistance is due to PTEN-mediated AKT regulation (Figure 4D). These results indicate that loss of PTEN can contribute to GC resistance in BCP-ALL.

But how does IKZF1 affect PTEN expression in BCP-ALL? To tackle this question, we compared protein expression of the transcriptional factor HES1 (hairy enhancer of split 1) in Nalm6 *IKZF1*^{KO} cells relative to control cells. HES1 is a known regulator of PTEN expression [20] and was shown to be critically involved in the regulation of GR-dependent gene expression [29]. Moreover, IKZF1 is known to directly repress HES1 expression [30]. Indeed, we observed that loss of IKZF1 leads to a strong increase of HES1 protein expression both in Nalm6 *IKZF1*^{KO} cells as well as Nalm6 and RS4;11 IKZF1 knockdown cells (Figure 5). In T-ALL, HES1 is controlled via activating mutations in NOTCH1 [20] and IKZF1 has been shown to repress NOTCH signaling in T cells [31] and to shape the repertoire of NOTCH target genes such as HES1 in T cells [32]. Thus, similar to the regulation of HES1 via NOTCH1 in T cells, we speculate that loss of IKZF1 leads to overexpression of HES1 in BCP-ALL.

Figure 4 Silencing of *PTEN* induces GC resistance in BCP-ALL cell line NALM6 (A) mRNA transcript levels of PTEN were measured in Nalm6 transduced with 2 independent *PTEN* shRNAs in comparison to non-targeting shRNA by quantitative reverse transcription PCR (qRT-PCR) and normalized to *Hprt* expression. **(B)** PTEN, AKT, p-AKT (Ser473), p-GR (Ser134) and GR protein expression levels of protein lysates from Nalm6 transduced with 2 independent *PTEN* shRNAs in comparison to non-targeting shRNA were analyzed by western blot. Tubulin was used as a loading control **(C)** Quantification of amine stainings measured in Nalm6 transduced with 2 independent *PTEN* shRNAs in comparison to non-targeting controls treated with 2μM Prednisolone for 48 hours. **(D)** Nalm6 transduced with 2 different *PTEN* shRNAs were treated for 48 hours with increasing concentrations of prednisolone in comparison to non-targeting shRNA with or without 0.5μM MK2206 and analyzed using an MTS based viability assay. All values were normalized to untreated Nalm6 cells. Error bars represent ± standard error of the mean (SEM). All experiments were repeated three times.


A
PTEN mRNA expression
Relative PTEN mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
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LUC shRNA
PTEN shRNA #1
PTEN shRNA #2
B
LUC shRNA
PTEN shRNA #1
PTEN shRNA #2
PTEN
pAKT (Ser473)
AKT
pGR (Ser134)
GR
γ-Tubulin
C
Amine staining
Dead cells in %
100
80
60
40
20
0
**
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LUC shRNA
PTEN shRNA #1
PTEN shRNA #2
D
MTS
Relative cell viability
1.2
1.0
0.8
0.6
0.4
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P<0.001
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0.002
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LUC-shRNA
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+MK2206

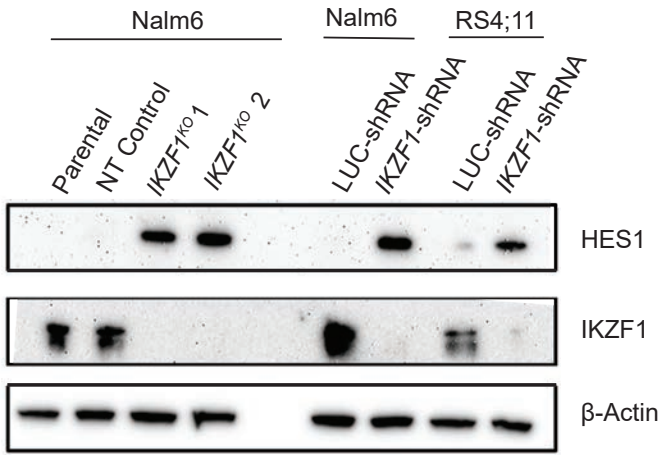
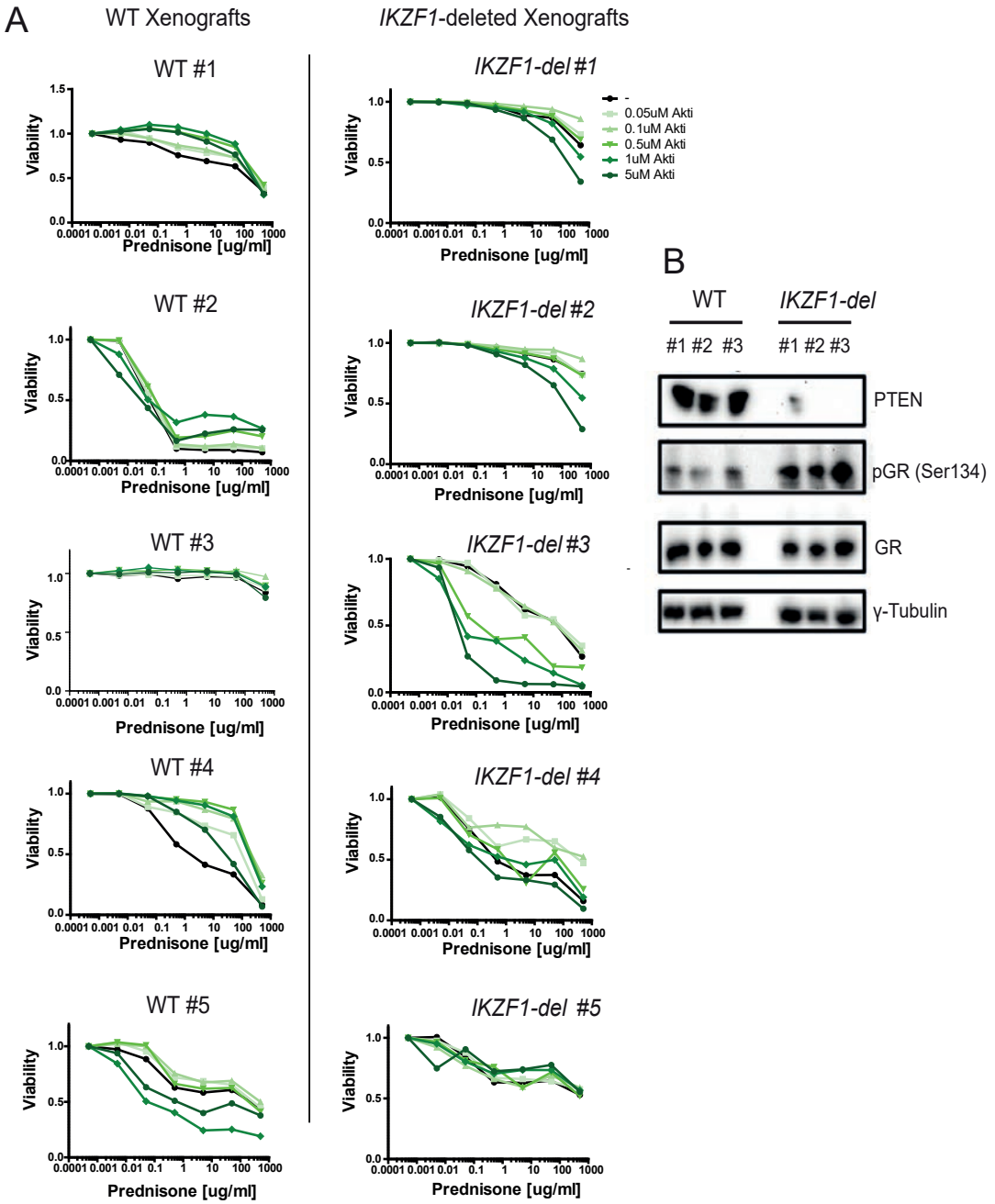


Figure 5 Loss of IKZF1 leads to increase in HES1 expression. HES1 protein expression of lysates from Nalm6 *IKZF1*^{KO} cells as well as Nalm6 and RS4,11 transduced with *IKZF1*-shRNA were analyzed by western blot. Respective non-targeting controls were used as control and actin was used as a loading control.

Figure 6 Patient-derived xenograft samples of primary BCP-ALL are resensitized towards GCs after Mk2206 treatment. hTERT immortalized MSCs were seeded in a 96 wells format and allowed to settle for 24 hours prior to the addition of BCP-ALL patient –derived xenografts (PDX). Cells were allowed to settle for 24 hours before prednisone was added in increasing concentrations with or without increasing concentrations of MK2206. (A) Prednisone sensitivity of 10 primary patient samples (5 wild-type vs. 5 *IKZF1*-deleted patient samples) after incubation with increasing concentrations of MK2206 and Prednisiolone after 3 days was analyzed by amine staining using flow cytometry. (B) Expression levels of PTEN, pGR(Ser134) and GR were analyzed in total protein lysates of three *IKZF1*–WT and three *IKZF1*-deleted PDX samples. Tubulin was used as a loading control.


A
WT Xenografts
IKZF1-deleted Xenografts
WT #1
IKZF1-del #1
WT #2
IKZF1-del #2
WT #3
IKZF1-del #3
WT #4
IKZF1-del #4
WT #5
IKZF1-del #5
Viability
Prednisone [ug/ml]
-
0.05uM Akti
0.1uM Akti
0.5uM Akti
1uM Akti
5uM Akti
B
WT
IKZF1-del
#1 #2 #3
#1 #2 #3
PTEN
pGR (Ser134)
GR
γ-Tubulin

AKT inhibition resensitized patient-derived xenografts to GC therapy

We next assessed the effect of AKT inhibition on GC resistance in primary BCP-ALL samples. For this, patient-derived xenografts (PDX) either with or without *IKZF1* deletion were co-cultured on MSCs and analyzed for GC response in the absence or presence of increasing concentrations MK2206. We performed a pilot screen with ten PDX samples in total (Five *IKZF1* WT samples versus five *IKZF1*-deleted samples). Out of ten samples, we found an effect of MK2206 treatment on GC sensitivity in four PDX samples. In detail, one out of five wild-type samples (WT#5) and one out of five *IKZF1*-deleted PDX samples (*IKZF1*-del #3) displayed a response to MK2206 treatment. Next to this, *IKZF1*-deleted samples #1 and #2 showed increased sensitivity to GCs but only under physiological very high concentrations of GCs (Figure 6A).

To test whether PTEN expression is affected in the PDX-samples, we performed Western blotting analysis comparing three available *IKZF1*-WT vs. three *IKZF1*-deleted PDX samples. We established that PTEN protein expression was reduced by 95% in *IKZF1*-deleted compared to *IKZF1*-WT PDX samples (Figure 6B). Because we detected high pGR Ser134 levels in GC resistant cells in our previous experiments, we also opted to correlate pGR Ser134 expression levels with PTEN protein expression and sensitivity to AKT inhibitors in this selection of PDX models. Indeed, loss of PTEN protein expression correlated with increased pGR (Ser134) levels (Figure 6B) in *IKZF1*-deleted PDX samples (2. fold to 4. fold increase in protein expression). Interestingly, the sample with the highest expression of pGR Ser134 (4. fold increase) was also effectively resensitized to GCs by AKT inhibition (*IKZF1*-del #3).

These data indicate that PDX samples with IKZF1 deletions showed a decrease in PTEN protein expression and hyperactivation of pGR134, but the reversal of GC resistance by an AKT inhibitor was only observed in part of the *IKZF1*-deleted PDX samples. Whether pGR134 expression levels could be an indicator for the success of AKT inhibition to break GC resistance in BCP-ALL needs to be studied in more detail in a larger patient cohort.

Discussion

IKZF1 aberrations can be found in approximately 15% of BCP-ALL [33,34] and have been identified as poor prognostic marker in BCP-ALL [15-18]. Moreover, loss of *IKZF1* affects therapy response in BCP-ALL. For instance, in *BCR-ABL1* positive ALL loss of *IKZF1* appears to promote integrin-dependent survival signaling through activation of focal adhesion kinase [27]. Indeed, FAK inhibition potentiated the responsiveness to ABL inhibitor dasatinib in a xenograft model [35]. Following our initial finding that *IKZF1* confers resistance to GCs [14], several studies have observed resistance to GCs in *IKZF1*-deleted ALL [36,37]. However, the molecular mechanism underlying this GC resistance has remained largely unknown. In this study, we identified inactivation of PTEN by the

transcriptional repressor HES1, a member of the basic helix-loop-helix transcription factor family and known target of IKZF1 [30] as a possible mechanism responsible for GC resistance in *IKZF1*-deleted BCP-ALL. Consistent with a loss of PTEN activity, we observed that deletion of *IKZF1* leads to activation of AKT and phosphorylation of the GR at Ser134, resulting in repression of GR mediated gene expression. Importantly, we observed that inhibition of AKT re-sensitized both *IKZF1*-deleted leukemia cell lines and, to a limited extend, also primary BCP-ALL cells to GC-induced apoptosis, by reducing GRSer134 phosphorylation to baseline levels.

Previous studies have demonstrated that phosphorylation of the GR on Serine 134 impairs translocation of the GR to the nucleus, thereby inhibiting the GR response [8].We were able to observe the same effect in murine B cells isolated from *Ikzf1*$^{+/-}$ mice compared to wild-type mice. However, diminished nuclear transport of the GR is probably not the only explanation for decreased GR activity. Other studies indicate that phosphorylation of Ser134 of the GR acts as general stress signal in BCP-ALL. This direct effect on the GR activity makes it arguable whether only shuttling of the GR, or also other stress routes may contribute to GC resistance [28]. It has been suggested that loss of *IKZF1* has a direct effect on GR expression levels [38], however neither previously [14] or in the current study did we observe changes in GR mRNA or protein expression of the GR as a result of *IKZF1* deletion.

Genetic aberrations leading to loss of PTEN function are common to many solid tumors [39], and can be detected in about 10% of T-ALL cases, but not in BCP-ALL. In T-ALL and solid tumors, inactivation of PTEN has been reported to lead to hyperactivation of the AKT pathway, leading to increased survival of *PTEN*-deleted cells [40]. Because of these observations, PTEN is generally considered a tumor suppressor. In contrast, PTEN has been suggested to have oncogenic effects in BCP-ALL [41]. However, shRNA-mediated knockdown of *PTEN* performed in our study did not negatively affect viability of BCP-ALL cell line NALM6 and protected cells from GC-induced apoptosis. This ambiguous role of PTEN in BCP-ALL should encourage further studies into the role of PTEN and the AKT route in BCP-ALL. Consistent with our findings, another study found that loss of PTEN has a potential protective role against GCs in BCP-ALL cells [42].

Our observations related to HES1 upregulation in response to loss of IKZF1 are in line with previous findings in T-ALL, where aberrated NOTCH1 signaling leads to upregulation of HES1 and suppression of PTEN [20,30]. We propose a similar model for BCP-ALL, in which HES1 is upregulated as a consequence of loss of IKZF1, a known repressor of HES1. It needs to be determined whether IKZF1 directly affects HES1 expression and whether we can link HES1-mediated regulation of GR to GC resistance in *IKZF1*-deleted BCP-ALL. Future chromatin immunoprecipitation experiments will help to shed light on question whether IKZF1 directly regulates PTEN, or through HES1, or whether other unknown factors are involved. For example, we cannot exclude that expression of NOTCH is deregulated in BCP-ALL due to loss of IKZF1, as loss of IKZF1 has been reported to lead to lineage infidelity in BCP-ALL [43].

Because of our limited collection of PDX samples and primary patient material available for cell viability assays and western blotting, this precludes any firm conclusions on the importance of this resistance mechanism in the patient. It is important to note that in this study as well as in previously reports from our group [14] not all patient samples with *IKZF1* deletions showed a similar poor response to GC-induced apoptosis. These findings suggest that threshold levels of IKZF1 expression itself, the type of *IKZF1* gene lesions as well as specific cell-intrinsic and genetic factors may contribute to the resistance phenotype in leukemic cells.

However, as GC sensitivity could be restored by AKT inhibition in all *IKZF1*-deleted BCP-ALL cell lines and in at least part of the PDX samples, this approach could potentially represent a new therapeutic option to break GC resistance in *IKZF1*-deleted BCP-ALL. In *IKZF1*-deleted patients showing resistance to GC therapy, phosphorylation status of the GR may be used as a biomarker to identify patients in which AKT inhibition could improve GC therapy response. However, for a better understanding of the relationship between genetic aberrations targeting *IKZF1*, phosphorylation of the GR, and the correlation with GC sensitivity, a larger cohort of PDX and primary patients will have to be studied. It would be valuable to perform phospho- flowcytometric analysis of pGR(Ser134) in a larger cohort of *IKZF1*-deleted leukemia to confirm the value of pGR(Ser134) as biomarker for combination of MK2206 and GCs. In the last years, 2nd and 3rd generation AKT inhibitors are entering clinical trials, which would be interesting to test in our PDX model systems to assess whether these novel inhibitors would be beneficial for the patient [44].

Taken together, our data indicate that *IKZF1*-mediated regulation of HES1 and PTEN represents a novel GC resistance route in BCP-ALL, while inhibition of AKT signaling may be effectively used to overcome GC resistance in part of the *IKZF1*-deleted ALLs.

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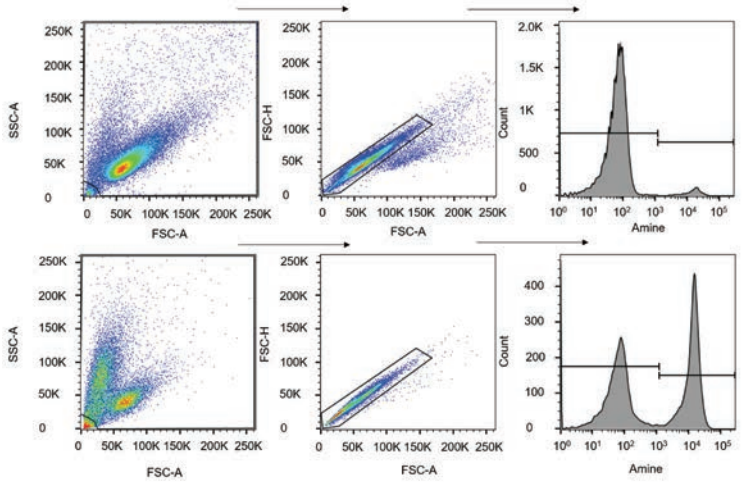
Supplemental Information Chapter 4

Supplementary Table 1 Primer sequences for quantitative reverse transcription PCR, IKZF1 guide RNAS and shRNA-vectors

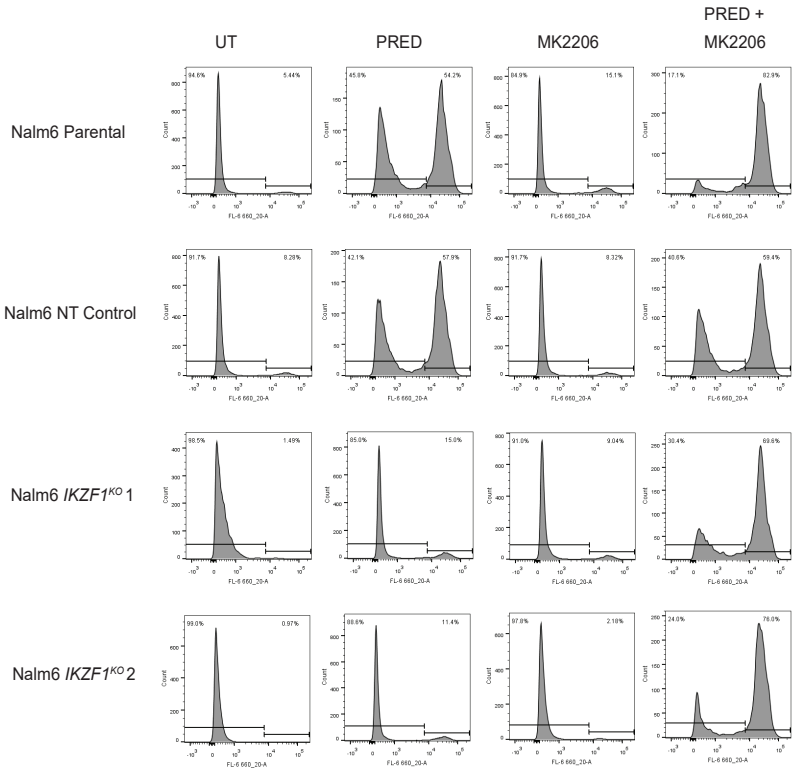
qPCR	Targets	Forward (5' -> 3')	Reverse (3' -> 5')
	PTEN	AAGGGACGAACTGGTGTAATG	GCCTCTGACTGGGAATAGTTAC
	IKZF1	ATGGATGCTGACGAGGGTCAAGAC	CTCTTGGAGCTTTGCTGTCCTCCC
	ZFP36L2	CACACTTCTGTCCGCCTTCTA	GCATGTTGTTCAGGTTGAGGT
Human	TSC22D3	CCAGCGTGGTGGCCATAGAC	GGATCTGCTCCTTCAGGATCTCCA
	NR3C1	CTGGGGACTCTGAACTTCCCTG	CTGTTGTTGCTGTTGAGGAGCTGG
	HPRT	GGTCCTTTTCACCAGCAAGCT	TGACACTGGCAAAACAATGCA
qPCR	**Targets**	**Forward (5' -> 3')**	**Reverse (3' -> 5')**
Mouse	Pten	ACACCGCCAAATTTAACTGC	GATTGTCATCTTCACTTAGCCATTG
	Hprt	GGGGGCTATAAGTTCTTTGCTGACC	TCCAACACTTCGAGAGGTCCTTTTCAC
shRNA	**Targets**	**Forward (5' -> 3')**	**Reverse (3' -> 5')**
Human	IKZF1	GAAGAATGTGCGGAGGATTT	AAATCCTCCGCACATTCTTC
	PTEN #1	AGGCGCTATGTGTATTATTAT	ATAATAATACACATAGCGCCT
	PTEN #2	CCACAGCTAGAACTTATCAAA	TTTGATAAGTTCTAGCTGTGG
	Control	CGTACGCGGAATACTTCGA	TCGAAGTATTCCGCGTACG
CRISPR gRNA	**Targets**	**Forward (5' -> 3')**	**Reverse (3' -> 5')**
Human	Control	CACCGGTAGCGAACGTGTCCGGCGT	AAACACGCCGGACACGTTCGCTACC
	IKZF1	CACCGCTCCAAGAGTGACAGAGTCGT	AAACACGACTCTGTCACTCTTGGAGC

Supplementary Table 2 Antibodies used in this studie

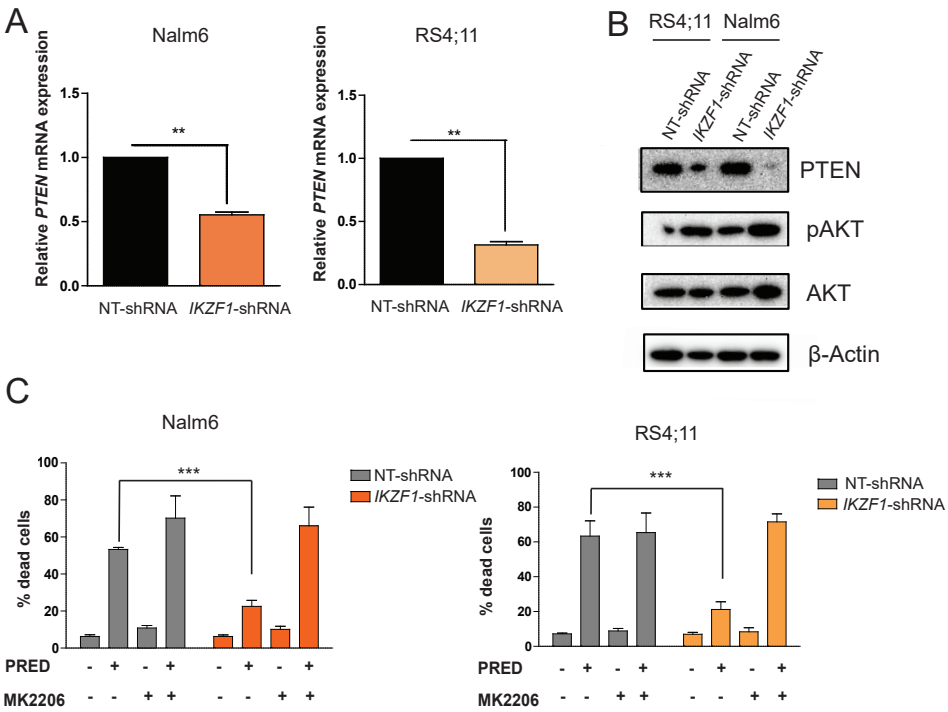
Primary Antibody	Dilution	Buffer	Reference	Provider	Secondary antibody
AKT	1:1000	BSA	9272	Cell Signaling	Anti-Rabbit HRP
p-AKT	1:1000	BSA	9271	Cell Signaling	Anti-Rabbit HRP
GR	1:1000	BSA	12041S	Cell Signaling	Anti-Rabbit HRP
p-GR (Ser134)	1:1000	BSA	ABS1008	Merck Millipore	Anti-Rabbit HRP
PTEN	1:1000	ELK	9188	Cell Signaling	Anti-Rabbit HRP
IKZF1	1:1000	ELK	AF4984	R&D systems	Anti-Goat HRP
Histone H3	1:2000	ELK	2650S	Cell Signaling	Anti-Rabbit HRP
HES1	1:1000	BSA	11988S	Cell Signaling	Anti-Rabbit HRP
Actin	1:10000	ELK	A5441	Sigma	Anti-Mouse HRP
Tubulin	1:10000	ELK	T6557	Sigma	Anti-Mouse HRP



Supplemental Figure 1 Representative gating strategy of primary xenografted patient samples on an MSC co-culture. After gating out debris from the viable cell population and the exclusion of doublets, population was further analyzed by amine staining.



Supplemental Figure 2 Representative analysis of Nalm6 *IKZF1*^{KO} cells after amine staining. Nalm6 non-targeting controls or *IKZF1*^{KO} cells treated with 2μM Prednisolone with or without 0.5μM MK2206 were analyzed by flow cytometry after amine staining



Supplemental Figure 3 shRNA mediated-IKZF1 knockdown cell lines show diminished PTEN mRNA expression and their GC sensitivity can be restored by AKT inhibtion. (A) mRNA transcript levels of PTEN were measured in Nalm6 transduced with *IKZF1*-shRNA and RS4,11 transduced with *IKZF1*-shRNA in comparison to non-targeting controls by quantitative reverse transcription PCR (qRT-PCR) and normalized to *Hprt* expression. (B) PTEN, AKT and p-AKT (Ser473) protein expression levels of protein lysates from Nalm6 and RS4;11 transduced IKZF1-shRNA in comparison to non-targeting shRNA were analysed by western blot. Actin was used as a loading control (C) Quantification of Amine stainings measured in Nalm6 transduced with *IKZF1*-shRNA (left panel) and RS4;11 transduced with *IKZF1*-shRNA (right panel) in comparison to non-targeting controls treated with 2μM Prednisolone with or without 0.5uM MK2206. All experiments were repeated three times.

Chapter 5

Tumor suppressors BTG1 and IKZF1 cooperate during mouse leukemia development and increase relapse risk in B cell precursor acute lymphoblastic leukemia patients

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Abstract

Deletions and mutations affecting lymphoid transcription factor IKZF1 (IKAROS) are associated with an increased relapse risk and poor outcome in B cell precursor acute lymphoblastic leukemia. However, additional genetic events may either enhance or negate the effects of *IKZF1* deletions on prognosis. In a large discovery cohort of 533 childhood B cell precursor acute lymphoblastic leukemia patients, we observed that single copy losses of *BTG1* were significantly enriched in *IKZF1*-deleted B cell precursor acute lymphoblastic leukemia (*P*=0.007). While BTG1 deletions alone had no impact on prognosis, the combined presence of *BTG1* and *IKZF1* deletions was associated with a significantly lower 5-year event-free survival (P=0.0003) and higher 5-year cumulative incidence of relapse (*P*=0.005) when compared with *IKZF1*-deleted cases without *BTG1* aberrations. In contrast, other copy number losses commonly observed in B cell precursor acute lymphoblastic leukemia, such as *CDKN2A/B*, *PAX5*, *EBF1* or *RB1*, did not affect outcome of *IKZF1*-deleted acute lymphoblastic leukemia patients. To establish whether the combined loss of IKZF1 and BTG1 function cooperate in leukemogenesis, *Btg1*-deficient mice were crossed onto an *Ikzf1* heterozygous background. We observed that loss of *Btg1* increased the tumor incidence of *Ikzf1*$^{+/-}$ mice in a dose-dependent manner. Moreover, murine B cells deficient for *Btg1* and *Ikzf1*$^{+/-}$- displayed increased resistance against glucocorticoids, but not to other chemotherapeutic drugs. Together, our results identify *BTG1* as a tumor suppressor in leukemia that, when deleted, strongly enhances relapse risk in *IKZF1*-deleted B cell precursor acute lymphoblastic leukemia and augments the glucocorticoid resistance phenotype mediated by loss of IKZF1 function.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common form of cancer in children and is characterized by recurrent genetic aberrations and chromosomal abnormalities, which represent distinct genetic subtypes that are used for risk stratification. [1] In the past, we and others have demonstrated that genomic alterations affecting the lymphoid transcription factor gene IKZF1 represent a strong prognostic factor associated with relapse in childhood B cell precursor ALL (BCP-ALL). [2-5] Recently, we established that loss of IKZF1 affects glucocorticoid (GC)-mediated gene regulation and confers GC resistance in BCP-ALL. [6] Deletion of *IKZF1* is a hallmark of *BCR-ABL1*-positive BCP-ALL, [7] and within this high-risk cytogenetic subtype, IKZF1 loss is associated with an even worse outcome. [8] *IKZF1* gene lesions are also frequently present in high-risk leukemias with a Philadelphia- or *BCR-ABL1*-like expression signature, [2,5,9] which often carry activated tyrosine kinases (i.e. *ABL1*, *PDGFRB*, *JAK2*, *FLT3*) or mutations targeting the RAS pathway. [10-12] Mouse studies have confirmed that two of these genomic alterations, namely *BCR-ABL1* and mutant *RAS*, cooperate with loss of IKZF1 during leukemic transformation. [13,14] On the other hand, *ERG* gene deletions constitute a specific subtype of BCP-ALL with favorable outcome, despite the frequent co-occurrence of *IKZF1* deletions. [15,16] Thus, specific genetic interactions may modulate the tumor suppressive functions of IKZF1 during leukemia development at initial diagnosis and at relapse after chemotherapy treatment.

Besides *IKZF1*, many other recurrent genetic aberrations have been observed in BCP-ALL, which include deletions affecting *BTG1*, *CDKN2A/B*, *EBF1*, *ETV6*, *PAX5*, *RAG1*, *RB1* and *TCF3*. [17-19] For some of these co-occurring genetic lesions, synergistic effects have been reported on leukemia development. For example, loss of Rag1 was shown to accelerate the onset of B cell lymphoblastic leukemia in *Cdkn2a/p19Arf*-deficient mice, [20] while increasing the incidence of T cell lymphomas in *Tcf3*$^{-/-}$ mice. [21] Furthermore, combined heterozygosity for *Ebf1* and *Pax5* results in a strong increase in the frequency of pro-B cell leukemia in mice. [22]

Here we describe the impact of deletions affecting the transcriptional coregulator B cell translocation gene 1 (*BTG1*), in co-occurrence with *IKZF1* loss on both leukemogenesis as well as outcome. We demonstrate that *BTG1* deletions are enriched in *IKZF1*-deleted pediatric BCP-ALL cases, and correlate with increased relapse risk in this patient group. Using mouse knockout models, we further demonstrate that loss of *Btg1* cooperates with *Ikzf1*$^{+/-}$ in the onset of ALL. Finally, our data indicate that both BTG1 and IKZF1 are important determinants of the GC therapy response and that combined loss of these tumor suppressors enhances GC resistance.

Methods

Clinical samples

The discovery cohort comprised of 533 pediatric patients with newly diagnosed BCP-ALL from three consecutive Dutch Childhood Oncology Group trials (DCOG ALL-8, ALL-9 and ALL-10) and two German Cooperative ALL trials (COALL 06-97 and 07-03). The validation cohort consisted of 515 pediatric patients enrolled in the Australian and New Zealand Children's Oncology Group (ANZCHOG) ALL8 protocol. In accordance with the Declaration of Helsinki, written informed consent was obtained from parents or legal guardians, and institutional review boards approved the use of excess diagnostic material for research purposes. Details on the patient cohorts, treatment regimens and outcomes, were described previously. [5,23,24]

Statistics patient data

Cumulative incidence of relapse (CIR) was estimated using a competing risks model, equality of CIRs was tested with Gray's test. Relapse and non-response to induction chemotherapy were considered as events, with death and secondary malignancy as competing events. Event-free survival (EFS) was calculated with non-response, relapse, secondary malignancy and death considered as events. EFS probabilities were estimated using the actuarial Kaplan-Meier method and survival data between groups were compared using univariate and multivariate Cox regression. The proportions of patients with *IKZF1* and other B cell development gene deletions as well as other categorical variables were compared using the Fisher's exact test. All P-values are two-sided, and a significance level of 0.05 or less was considered to be significant. Analyses were performed in R 3.0.1 (2013-05-16), using the packages cmprsk version 2.2-7, mstate version 0.2.7, and survival version 2.37-4. [25-27]

Mice

Btg1 and *Ikzf1* (Ik^{Neo}) knockout lines have been described previously, [28,29] and were intercrossed on a *C57Bl/6J* background. Mice were maintained under specific pathogen-free conditions at our Central Animal Laboratory facility. Genotyping of the offspring was performed by PCR (primer sequences are listed in *Online Supplementary Table S1*). Animal experiments were approved by the Animal Experimental Committee of the Radboud university medical center and were performed in accordance with institutional and national guidelines.

Functional characterization murine lymphocytes

Detailed information on functional characterization of normal and leukemic lymphocytes by flow cytometry, immunohistochemistry, IG/TR PCR and cell viability assays can be

found in the *Online Supplementary Methods* section. For analyzing the glucocorticoid response in B-lymphocytes, mononuclear splenocytes were isolated from wild-type and the different knockout mice and stimulated *in vitro* with 5 µg/mL lipopolysaccharide (LPS) for 48 hours. The obtained activated B-lymphocytes (≥ 80% B220+) were isolated by ficoll gradient and cultured for another 48 hours in the absence or presence of the synthetic glucocorticoids prednisolone or dexamethasone. Thereafter, relative cell viability was assessed by MTS assay and AnnexinV/7-AAD staining.

Results

BTG1 deletions are enriched in *IKZF1*-deleted pediatric BCP-ALL

Gene deletion of the tumor suppressor *IKZF1*, creating either dominant-negative IKZF1 isoforms or haploinsufficiency, is an important predictor of poor outcome in BCP-ALL, [2-5] but to what extent other additional common single gene deletions, such as *CDKN2A/B*, *PAX5*, *BTG1*, *ETV6*, *EBF1* or *RB1* impact the prognostic value of *IKZF1* has not been clearly established. To address this question, we studied a previously described childhood BCP-ALL cohort of 533 cases enrolled in consecutive DCOG and COALL trials. [5] The representation of the different BCP-ALL subtypes was similar between the DCOG and COALL cohorts, and comparable to that described in literature (*Online Supplemental Table S2*). We identified 105 BCP-ALL patient samples containing an *IKZF1* deletion. Within the *IKZF1*-deleted group, we observed a significant enrichment for *BTG1* deletions (P=0.007), where 17 of the 105 *IKZF1*-deleted cases (16%) harbored *BTG1* deletions as compared to 31

Table 1 Co-occurrence of *IKZF1* deletion with other common gene deletions in BCP-ALL

	***IKZF1* no deletion**		***IKZF1* deletion**		**Fisher Test**	
	n	**%**	**n**	**%**	***P*-value**	**Odds ratio**
BTG1 no deletion	397	0.93	88	0.84	0.0071	2.5
BTG1 deletion	31	0.07	17	0.16		
PAX5 no deletion	330	0.77	55	0.52	1.40E-06	3.1
7 deletion	98	0.23	50	0.48		
CDKN2A/B no deletion	298	0.70	53	0.50	3.40E-04	2.2
CDKN2A/B deletion	130	0.30	52	0.50		
EBF1 no deletion	405	0.95	96	0.91	0.25	1.6
EBF1 deletion	23	0.05	9	0.09		
RB1 no deletion	400	0.93	91	0.87	0.026	2.2
RB1 deletion	28	0.07	14	0.13		
ETV6 no deletion	296	0.69	80	0.76	0.19	0.7
ETV6 deletion	132	0.31	25	0.24		

of the 397 *IKZF1*-wild type cases (7%) (Table 1). These focal *BTG1* deletions mainly covered the second exon of *BTG1* and downstream adjacent sequences as described previously. [30] Similarly, deletions affecting *PAX5* (*P*<0.0001), *CDKN2A/B* (*P*=0.0003) and *RB1* (*P*=0.026) were present at higher frequencies in *IKZF1*-deleted cases, while this was not observed for *EBF1* or *ETV6* deletions (Table 1). To the converse, in the *BTG1*-deleted group we observed significant enrichment for *IKZF1* (*P*=0.007), *EBF1* (*P*=0.0011), *RB1* (*P*=0.042), and *ETV6* (*P*=0.0046) deletions (*Online Supplemental Table S3*), which is in agreement with our previous findings. [30] Previously, it has been shown that both *IKZF1* and *BTG1* deletions are strongly enriched in the cytogenetic *BCR-ABL1* subtype. [7,31] Consistent with this notion, we observed that of 24 *BCR-ABL1*-positive cases 7 (29%) harbored deletions in both *IKZF1* and *BTG1* (Table 2), which represents 47% of *IKZF1*-deleted and 100% of the *BTG1*-deleted cases found in the *BCR-ABL1*-positive group (Table 2).

Table 2 Pediatric BCP-ALL patient characteristics in the *BTG1*- and *IKZF1*-single and *BTG1;IKZF1*–double deleted groups

Patients' characteristics	***BTG1*-del; *IKZF1*-wt**		***BTG1*-wt; *IKZF1*-del**		***BTG1*-del; *IKZF1*-wt**		***BTG1*-wt; *IKZF1*-wt**		**Total**		**Fisher *P*-value**
	n=17 (3%)		n=88 (20%)		n=31 (6%)		n=397 (74%)		n=533 (100%)		
	n	%	n	%	n	%	n	%	n	%	
Gender											0.3
Female	5	29%	44	50%	11	35%	183	46%	243	46%	
Male	11	71%	44	50%	20	65%	214	54%	290	54%	
Age (years)											0.0054
< 10	11	65%	60	68%	26	84%	330	83%	427	80%	
≥ 10	6	35%	28	32%	5	16%	67	17%	106	20%	
WBC (cells/nl)											0.066
< 50	11	65%	55	62.5%	23	74%	299	76%	388	73%	
≥ 50	6	35%	33	37.5%	8	26%	96	24%	143	27%	
NCI-Rome											0.016
SR	8	47%	39	44%	19	61%	245	62%	311	58%	
HR	9	53%	49	56%	12	39%	151	38%	221	42%	
Protocol											0.54
DCOG	14	82%	63	72%	25	81%	311	78%	413	77%	
COALL	3	18%	25	28%	6	19%	86	22%	120	23%	
Subtype											
ETV6-RUNX1	2	12%	2	2%	24	77%	126	32%	154	29%	<0.0001
HeH	1	6%	16	18%	2	7%	107	27%	126	24%	0.0055
B-other	4	24%	23	26%	5	16%	75	19%	107	20%	0.41
BCR-ABL1-like	3	18%	38	43%	0	0%	51	13%	92	17%	<0.0001
*TCF3*r	0	0%	0	0%	0	0%	19	5%	19	4%	0.093
BCR-ABL1	7	41%	8	9%	0	0%	9	2%	24	5%	<0.0001
*MLL*r	0	0%	1	1%	0	0%	10	3%	11	2%	0.89

del, deletion; WBC, white blood cell count; NCI-Rome HR, high risk defined by age at diagnosis ≥ 10 years and/or WBC ≥ 50 cells/nl; HeH, high hyperdiploid (51-65 chromosomes); P-value, Fisher exact test across the four deletions groups; WBC for 2 cases missing, NCI Risk for 1 case missing, both in the BTG1-*wt* ;IKZF1-*wt group.*

Combined deletions of *BTG1* and *IKZF1* predict inferior outcome in BCP-ALL

We next compared clinical characteristics of *BTG1;IKZF1* double-deleted cases, *IKZF1* and *BTG1* only-deleted cases, and cases without *IKZF1* or *BTG1* deletion in our complete childhood BCP-ALL cohort. The characteristics of these four groups were similar to the total cohort with respect to gender and treatment protocol (Table 2). The double-deleted and *IKZF1* only-deleted groups contained more patients older than 10 years of age and increased white blood cell counts, and hence more NCI-Rome high-risk cases [32] (Table 2). We compared the event-free survival (EFS) and cumulative incidence of relapse (CIR) between cases with both *IKZF1* and *BTG1* deletions (*BTG1*-del;*IKZF1*-del, n=17) with *IKZF1*-only deleted cases (*BTG1*-wt;*IKZF1*-del, n=88) (Figure 1A-B). The 5-year CIR in *IKZF1* plus *BTG1*-deleted cases was 53% ± 13% compared with 28% ± 5% in *IKZF1* only-deleted cases (*P*=0.005; Table 3A). Similarly, the 5-year EFS was lower in double-deleted cases compared with *IKZF1* only-deleted cases (HR 3.5, *P*=0.0003; Table 3A). In contrast, the *BTG1* only-deleted cases (*BTG1*-del;*IKZF1*-wt, n=31) showed a similar outcome (5-year CIR:10% ± 6%) as the reference cases without *IKZF1* or *BTG1* deletions (n=397; 5-year CIR:13% ± 2%). The synergistic effect of loss of *BTG1* and *IKZF1* on outcome remained after correction for subtype in the Cox model (Table 3A) and after leaving out the *BCR-ABL1*-positive cases (Figure 1C-D; Table 3B). For the *BCR-ABL1*-positive cases, *BTG1* deletions did not further impact the poor treatment outcome as observed for the *IKZF1* only-deleted cases (Figure 1E-F; Table 3C).

As deletions of *PAX5*, *CDKN2A/B* and *RB1* were similarly enriched in *IKZF1*-deleted BCP-ALL, we examined the impact of these deletions on the outcome of *IKZF1*-deleted cases. In contrast to *IKZF1;BTG1* double-deleted patients, the outcome of patients with co-occurring *PAX5*, *CDKN2A/B* or *RB1* deletions did not differ from *IKZF1* only-deleted cases (Figure 2; Online Supplementary Table S4). Similarly, co-occurrences of *IKZF1* deletions with either *EBF1* or *ETV6* deletions did not affect outcome compared with *IKZF1* only-deleted. To validate our findings, we analyzed the Australian ANZCHOG ALL8 cohort (n=515) [23,24] to assess the prognostic value of *BTG1;IKZF1*-double deletions. In this cohort, 6 out of 11 *BTG1*-del;*IKZF1*-del patients developed a relapse (*Online Supplementary Figure S1*). The 5-year CIR in the *BTG1;IKZF1*-double deleted group was 61% ± 19% versus 35% ± 6% in the *IKZF1*-only deleted group (P=0.19; Table 3D). Hence, the same trend was observed in this independent validation cohort, albeit statistically non-significant. Together, these data indicate that *BTG1* deletions in an unselected leukemia population have no prognostic value, but *BTG1* copy number losses specifically exacerbate the effects of *IKZF1* deletion on inferior outcome in BCP-ALL.

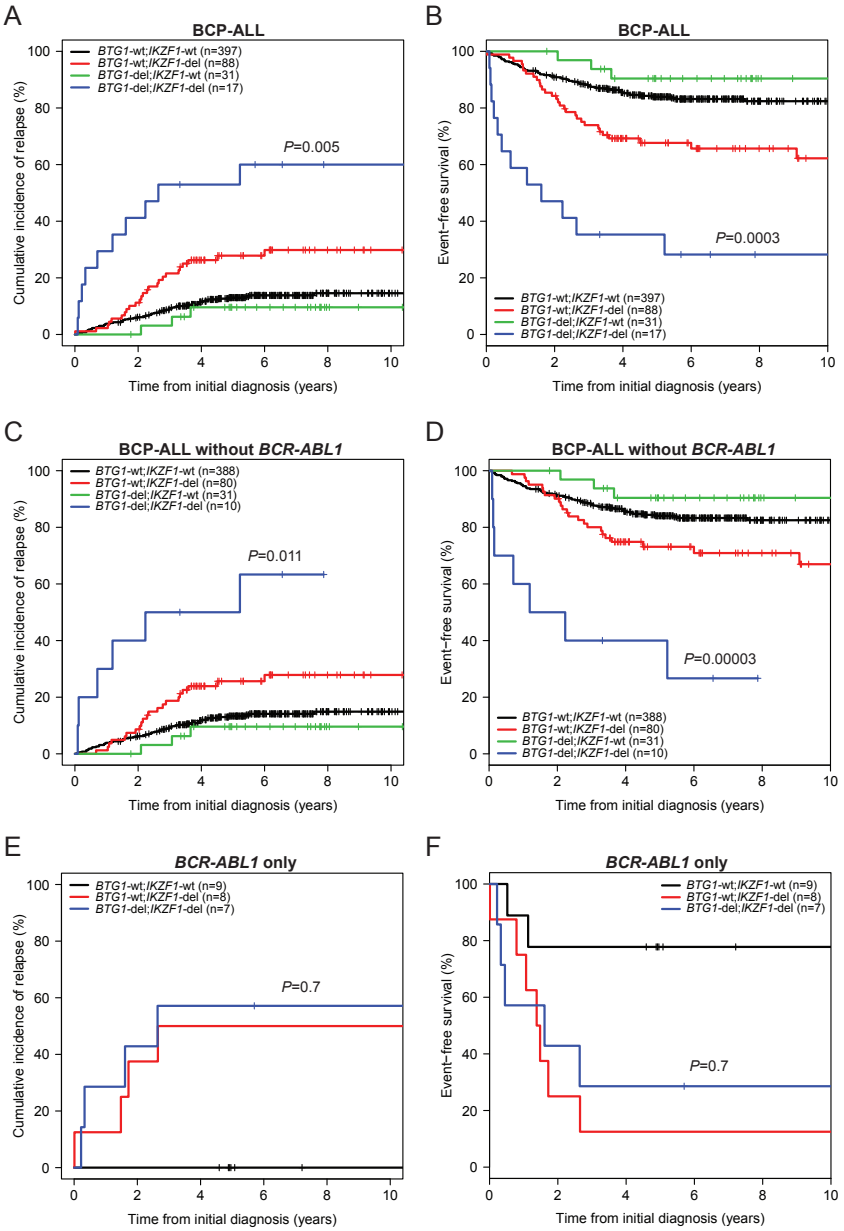

Figure 1 Cumulative incidence of relapse (CIR) and event-free survival (EFS) curves for pediatric BCP-ALL cases with or without *IKZF1* and *BTG1* deletions. **(A)** CIR and **(B)** EFS curves for total BCP-ALL cohort (n=533), *IKZF1* plus *BTG1* deletion, **(C)** CIR and **(D)** EFS curves for BCP-ALL without *BCR-ABL1*-positive cases (n=509), *IKZF1* plus *BTG1* deletion, **(E)** CIR (F) EFS curves for *BCR-ABL1*-positive cases (n=24), *IKZF1* plus *BTG1* deletion. Colors: black, *IKZF1* and *BTG1*-wildtype; green, *IKZF1*-wildtype, *BTG1*-deleted; red, *IKZF1*-deleted, *BTG1*-wildtype; blue, both *BTG1* and *IKZF1*-deleted. Abbreviations: wt, wildtype; del, deletion; n, total number. For CIR graphs **(A,C,E)** the Gray *P*-value and for the EFS graphs **(B,D,F)** the Cox P-value is indicated comparing *BTG1*-del;*IKZF1*-del with *BTG1*-wt;*IKZF1*-del.

Table 3 CIR and EFS analysis of *BTG1;IKZF1*-double deleted pediatric BCP-ALL cases compared with *IKZF1*-only deleted cases

A

BCP-ALL[1] (n=533)				Univariate				Multivariate	
Deletion	**Total**	**Relapse**	**Death**	**5-yr CIR (SE)**	**Gray P**	**EFS HR (95% CI)**	**Cox P**	**EFS HR (95% CI)**	**Cox P**
BTG1;IKZF1	17	10	2	53% (13%)	0.005	3.5 (1.8-6.8)	0.0003	2.5 (1.0-5.9)	0.043
IKZF1	88	25	5	28% (5%)					
BTG1	31	3	0	10% (6%)					
none	397	52	12	13% (2%)					

B

BCP-ALL[1] without BCR-ABL1 (n=509)				Univariate				Multivariate	
Deletion	**Total**	**Relapse**	**Death**	**5-yr CIR (SE)**	**Gray P**	**EFS HR (95% CI)**	**Cox P**	**EFS HR (95% CI)**	**Cox P**
BTG1;IKZF1	10	6	1	50% (17%)	0.011	4.4 (1.9-10.3)	0.0007	6.7 (2.7-16)	0.00003
IKZF1	80	21	2	26% (5%)					
BTG1	31	3	0	10% (6%)					
none	388	52	10	13% (2%)					

C

BCP-ALL[1] BCR-ABL1 only (n=24)				Univariate				Multivariate	
Deletion	**Total**	**Relapse**	**Death**	**5-yr CIR (SE)**	**Gray P**	**EFS HR (95% CI)**	**Cox P**	**EFS HR (95% CI)**	**Cox P**
BTG1;IKZF1	7	4	1	57% (22%)	0.7	0.8 (0.3-2.5)	0.7	ND	ND
IKZF1	8	4	3	50% (21%)					
BTG1	0	0	0						
none	9	0	2	0% (0%)					

D

BCP-ALL[2] validation cohort (n=515)				Univariate				Multivariate	
Deletion	**Total**	**Relapse**	**Death**	**5-yr CIR (SE)**	**Gray P**	**EFS HR (95% CI)**	**Cox P**	**EFS HR (95% CI)**	**Cox P**
*BTG1;IKZF1**	11	6	0	61% (19%)	0.19	01.83 (0.8-4.2)	0.16	ND	ND
IKZF1	69	23	4	35% (6%)					
*BTG1**	45	13	0	27% (7%)					
none**	390	55	4	13% (2%)					

[1]Discovery cohort: pediatric patients from consecutive DCOG/COALL trials; [2]Validation cohort: pediatric patients from ANZCHOG ALL8 trial; CIR, cumulative incidence of relapse; SE, standard error; EFS, event-free survival; HR, hazard ratio; CI, confidence interval; multivariate, corrected for BCP-ALL subtype and stratified for study cohort (DCOG, COALL). None: neither BTG1 *nor* IKZF1 *deletion. * Report of 1 secondary malignancy in these groups; ** Report of 6 independent secondary malignancies in this group.*

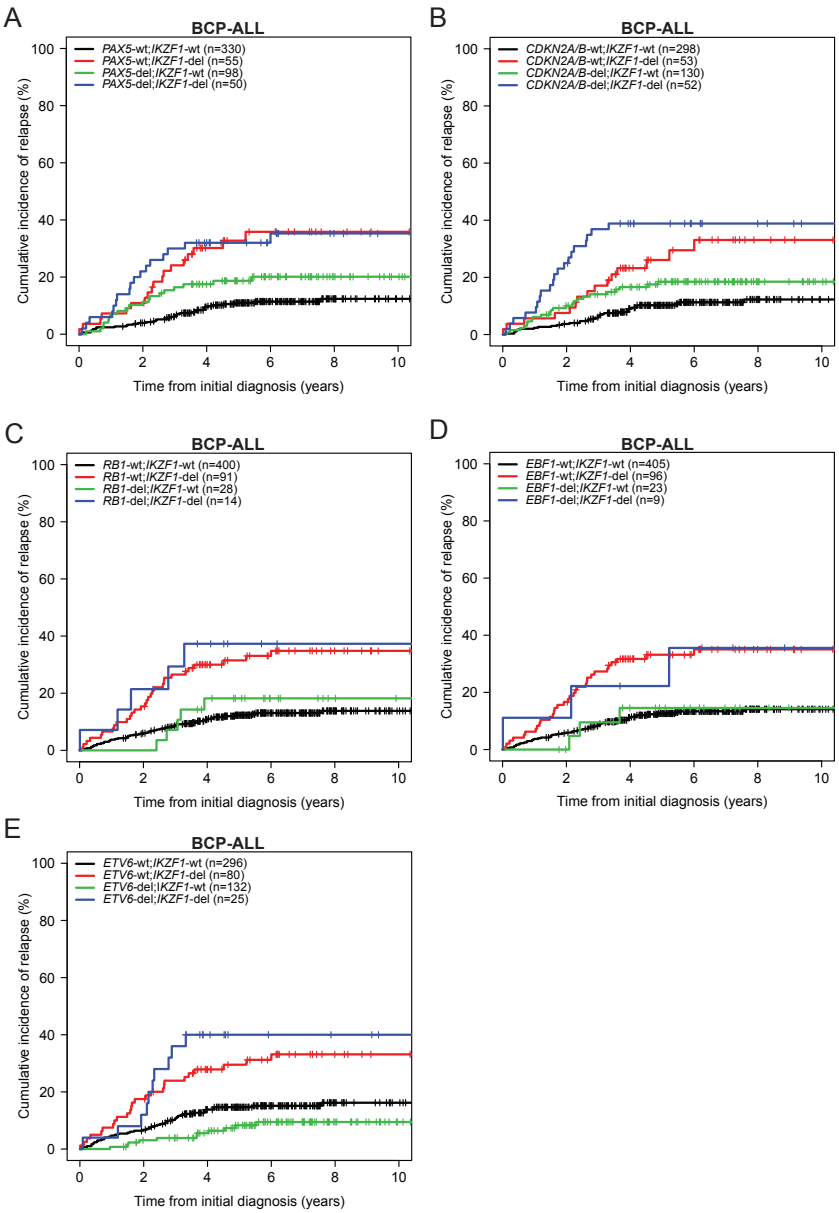

Figure 2 Cumulative incidence of relapse (CIR) curves for pediatric BCP-ALL cases with or without *IKZF1* deletions in combination with other single common gene deletions. **(A)** *IKZF1* plus *PAX5*, **(B)** *IKZF1* plus *CDKN2A/B*, **(C)** *IKZF1* plus *RB1*, **(D)** *IKZF1* plus *EBF1*, **(E)** *IKZF1* plus *ETV6* (n=533). Colors: black, none of the indicated deletions; green, *IKZF1*-wildtype, indicated gene deleted; red, *IKZF1*-deleted, indicated gene wildtype; blue, both *IKZF1* and indicated gene deleted. Abbreviations: wt, wildtype; del, deletion; n, total number.

Leukemia predisposition in *Btg1* knockout mice

Although deletions affecting the *BTG1* gene are a frequent event in BCP-ALL, a direct role for BTG1 in leukemia development has not been reported. Therefore, we first examined the tumor suppressive function of BTG1 using a constitutive *Btg1* knockout line harboring a Neo-cassette into the first coding exon. [28] We previously reported that these *Btg1* knockout mice display defective B cell development with a 25% reduction in the amount of progenitor B cells within the bone marrow compartment, mainly affecting the pre-B and immature B cell stage. [33] Furthermore, *Btg1* is required for optimal proliferative expansion of early progenitor B cells in methylcellulose in response to interleukin-7. At the same time, there was no obvious defect in the development of myeloid and T-lymphoid cells in these *Btg1*-deficient animals. [33] In this study, mice that carried either one or two copies of the *Btg1*-knockout allele were followed over a period of 18 months, along with control litter mates. About 6% (n=3/49) of the wild-type *C57BL6/J* mice developed B cell lymphomas between the age of 14 and 18 months (Table 4), which is consistent with previous observations. [34] Within the same time period 6% of the $Btg1^{+/-}$ (n=2/34) and 18% of the $Btg1^{-/-}$ (n=6/33) mice developed exclusively T cell leukemia (Table 4), characterized by enlarged primary lymphoid organs, such as spleen and lymph nodes, and focal infiltration of leukemic T cells into peripheral organs, such as lung and liver. These *Btg1*-deficient T cell leukemias expressed the T cell surface marker CD3, and displayed clonal T cell receptor (TR) rearrangements (Figure 3B). In addition, these $CD3^+$ T cell leukemias showed increased expression of the T cell activation marker CD44, but also large numbers of $B220^+$ cells within the infiltrated areas of tissues, such as liver or lung, and affected lymph nodes (*Online Supplementary Figure S2*). There was no evidence for clonal immunoglobulin gene rearrangements in these Btg1-/- T cell leukemias (Online Supplementary Figure S2), suggesting the presence of a substantial number of non-malignant B-lymphocytes in the proximity of these leukemic T cells. These data show that, although somatic BTG1 deletions predominantly occur in BCP-ALL, *Btg1*-deficiency in the mouse germline predisposes exclusively to T cell malignancies. This predilection for T-lineage leukemias is also observed for other knockout mouse models targeting genes commonly deleted in BCP-ALL, such as *Ikzf1* mutant mice. [35,36]

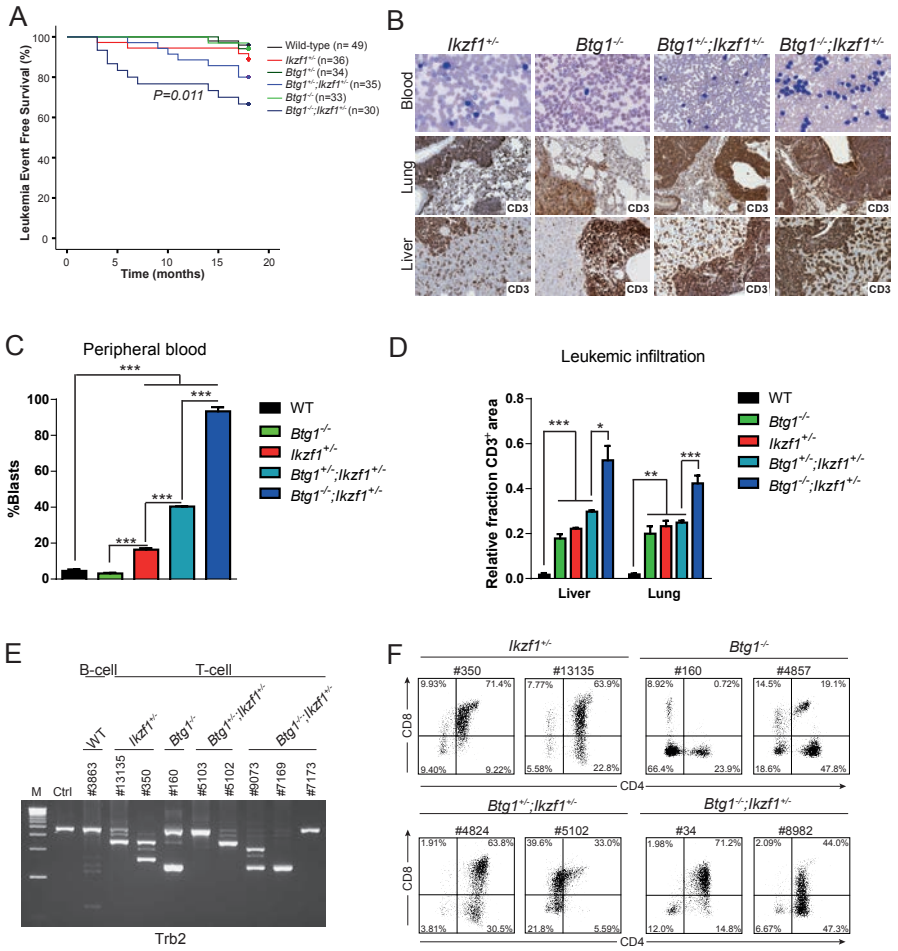

Figure 3 Leukemia incidence and phenotype of *Btg1* knockout mice intercrossed with haplodeficient *Ikzf1* animals. **(A)** Kaplan-Meier survival curve indicates the leukemia event free survival in wild-type, *Ikzf1*$^{+/-}$, *Btg1*$^{+/-}$, *Btg1*$^{+/-}$;*Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$, and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice over a time period of 17 months. Leukemia incidence is significantly increased in *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice as compared to *Ikzf1*$^{+/-}$ mice (*P*=0.011). **(B)** Peripheral blood smear stained with Giemsa and immunohistochemistry for CD3 on lung and liver tissues of diseased *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$, *Btg1*$^{+/-}$;*Ikzf1+*$^{/-}$, and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice. **(C)** Quantification of blast counts in peripheral blood smear stained with Giemsa of diseased wild-type, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$, *Btg1*$^{+/-}$;*Ikzf1*$^{+/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice (n=3). The percentage of leukemic blasts is indicated. **(D)** Quantification of T cell (CD3) infiltration into liver and lung of diseased wild-type, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$, *Btg1*$^{+/-}$;*Ikzf1*$^{+/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice (n=3) using FIJI software. Data are represented as the positively stained area divided by the total area measured, with standard errors of the mean P-values (two-sided t test). **P*<0.05, ***P*<0.01, and ****P*<0.001. **(E)** T cell receptor beta 2 (Trb2) gene rearrangement analysis by PCR on control tissue, B cell lymphoma derived from wild-type mice (#3863), and T cell leukemias derived from *Ikzf1*$^{+/-}$ (#13135, #350), *Btg1*$^{-/-}$ (#160), *Btg1*$^{+/-}$;*Ikzf1+/-* (#5103 and #5102) and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice (#9073, #7169, #7173). **(F)** Flow cytometry analyzing CD4 and CD8 expression on T cell leukemia samples of two *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$, *Btg1*$^{+/-}$;*Ikzf1*$^{+/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice.

Table 4 Characteristics of lymphoid tumors derived from single and intercrossed *Btg1* and *Ikzf1* knockout lines

Genotype	Tumor incidence (% mice)	Mean age (months)	Tumor phenotype	*Ikzf1*$^{+/-}$	*P*-value [a] *Btg1*$^{+/-}$	*Btg1*$^{-/-}$
Wild-type	3/49 (6%)	16.4	B cell lymphoma	-	-	-
Ikzf1$^{+/-}$	5/36 (14%)	12.4	T cell leukemia	-	0.271	0.703
Btg1$^{+/-}$	2/34 (6%)	15.8	T cell leukemia	0.271	-	0.143
Btg1$^{+/-}$;*Ikzf1*$^{+/-}$	10/35 (29%)	13.6	T cell leukemia	0.134	0.013	0.231
Btg1$^{-/-}$	6/33 (18%)	17.4	T cell leukemia	0.703	0.143	-
Btg1$^{-/-}$;*Ikzf1*$^{+/-}$	12/30 (40%)	9.4	T cell leukemia	0.011	0.001	0.026

[a] *Chi-square analysis comparing incidence of T cell leukemia to indicated genotype at 18 months*

Loss of *Btg1* increases leukemia incidence in *Ikzf1*$^{+/-}$ mice

To investigate cooperation between BTG1 and IKZF1 during leukemogenesis, we intercrossed Btg1-deficient mice with haplodeficient *Ikzf1* mice using the *Ik*^{Neo} mouse line, [29] which harbors a Neo-floxed knock-in allele combined with a Pax5-IRES-GFP cDNA at the first coding exon of *Ikzf1*, thereby creating an *Ikzf1*-null allele. [29] These mice are only viable as a heterozygous knockout line (*Ikzf1*$^{+/-}$). First, we analyzed the phenotype of young animals (age 6-12 weeks) to assess the effect of *Ikzf1*$^{+/-}$-allele on *Btg1*-deficiency in B- and T-lymphoid development. *Ikzf1*$^{+/-}$ mice, like *Btg1*$^{-/-}$ mice, displayed a moderate reduction in the fraction of B220^{+}-cells in bone marrow (BM) and spleen (*Online Supplementary Figure S3*). This correlated with a partial block at the pre-pro B cell stage (Hardy fraction A) and the pre-B cell stage (Hardy fraction D) in *Ikzf1*$^{+/-}$ mice (*Online Supplementary Figure S3*). *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice showed an even stronger reduction in B220^{+}-cells, with additive effects at both B220^{+}CD43^{+} and B220^{+}CD43^{-} differentiation stages in BM (Hardy fractions A to E) (*Online Supplementary Figure S3*). In contrast, *Btg1-/-;Ikzf1*$^{+/-}$ mice, similar to *Btg1*$^{-/-}$ and *Ikzf1*$^{+/-}$ single knockout animals, showed no major defects in post-natal thymic T cell development (*Online Supplementary Figure S4*).

Next, we followed *Ikzf1*$^{+/-}$, *Btg1*$^{+/-}$;*Ikzf1*$^{+/-}$ and *Btg1*$^{-/-}$*Ikzf1*$^{+/-}$ mice for a period of 18 months. In our cohort, 14% (n=5/36) of the *Ikzf1*$^{+/-}$ mice developed T cell leukemia between 3 and 18 months of age, with a median age of 12 months (Figure 3A; Table 4). Similar to *Btg1*-deficient leukemia, the *Ikzf1*$^{+/-}$ T cell leukemias showed infiltration of CD3^{+} leukemic blasts into distant organs and clonal TR rearrangements (Figure 3). Interestingly, we observed an increased leukemia incidence upon combined loss of *Btg1* and *Ikzf1* (Figure 3A), which is consistent with our hypothesis that *BTG1* deletions cooperate with *IKZF1* aberration to induce human BCP-ALL. We found that 26% of the *Btg1*$^{+/-}$;*Ikzf1*$^{+/-}$ mice (n=10/35) and 40% of the *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ (n=12/30) animals developed T cell leukemia, while leukemias in *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ appeared with a slightly shorter latency (9.4 months) relative to *Ikzf1*$^{+/-}$ mice (12.4 months) (*P*=0.011) (Table 4). Tumors in the *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ compound mice were

characterized by significantly higher leukocyte counts in peripheral blood compared to single knockout animals (Figure 3B and 3C), and strong infiltration of leukemic cells into liver and lungs (Figure 3B and 3D), as well as clonal TR rearrangements (Figure 3E). Flow cytometric analysis of the different T cell leukemias revealed that most of the *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ leukemias were CD4^{+}CD8^{+} double positive T cells with ongoing differentiation towards CD4 or CD8 single positive stage (Figure 3F, *Online Supplementary Table S5*). Moreover, isolated leukemic T cells, derived from all the different genetic backgrounds included in our studies (*Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$), could be serially transplanted into syngeneic *C57BL6/J* mice giving rise to similar (oligo)clonal T cell leukemias (*Online Supplementary Figure S5*). Taken together, our data demonstrate that loss of *Btg1* cooperates with haplodeficiency for *Ikzf1* during mouse leukemia development in a dose-dependent manner.

BTG1 modifies glucocorticoid resistance mediated by loss of IKZF1

While these experiments confirm the genetic interaction between *BTG1* deletions and IKZF1 aberrations during leukemogenesis, they do not explain the poor outcome observed in patients showing combined loss of *BTG1* and *IKZF1*. Recently, we established that inferior outcome related to *IKZF1* deletions in BCP-ALL is correlated with an attenuated *in vivo* day 8 prednisolone response and increased GC resistance in *IKZF1*-deleted primary leukemic cells, as determined by *in vitro* MTT assays. [6] These results could be recapitulated using primary splenic B cells isolated from I*kzf1*$^{+/-}$ mice, which revealed that also non-leukemic *Ikzf1*$^{+/-}$ B cells are less sensitive towards GC-induced apoptosis. [6] Based on our previous findings that BTG1 regulates glucocorticoid receptor activation, [37] we investigated whether loss of BTG1 would impact the GC response in primary murine B cells. To this end, B cells isolated from WT, *Btg1*$^{-/-}$, *Ikzf1*$^{-/-}$ and *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ mice and obtained after lipopolysaccharide activation, were stimulated for 48 hours with increasing concentrations of prednisolone or dexamethasone and subjected to MTS assays to assess relative cell survival. While *Btg1*-deficiency alone had no effect on GC-induced apoptosis, *Ikzf1*-haplodeficient B cells showed enhanced cell survival as compared to WT (Figure 4A), similar to our previous findings. [6] Importantly, *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ B cells showed an even stronger resistance to GC-induced apoptosis when compared to *Ikzf1*$^{+/-}$ B cell (P<0.001). These findings were confirmed by Annexin V staining, demonstrating a significantly smaller apoptotic fraction in *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ B cells relative to *Ikzf1*$^{+/-}$ B cells (P<0.001) (Figure 4B). Analyses of primary *Btg1*$^{-/-}$, *Ikzf1*$^{+/-}$ and *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ thymocytes revealed no differential sensitivity to GC-induced apoptosis as compared to WT (*Online Supplementary Figure S6A*). Next, we assessed whether loss *Btg1* and *Ikzf1* would promote resistance in B cells to other chemotherapeutic drugs commonly used in the treatment of BCP-ALL patients, including 6-mercaptopurine, doxorubicin, vincristine and asparaginase. However, *Btg1*$^{-/-}$, *Ikzf1*$^{+/-}$ and *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ B cells showed similar cell survival in comparison to control cells (*Online Supplementary Figure S6B*). Together, these data argue that loss of tumor suppressor BTG1 enhances GC resistance in the context of *IKZF1*-deletions, which may explain the inferior treatment outcome observed in patients showing combined loss of *BTG1* and *IKZF1*.

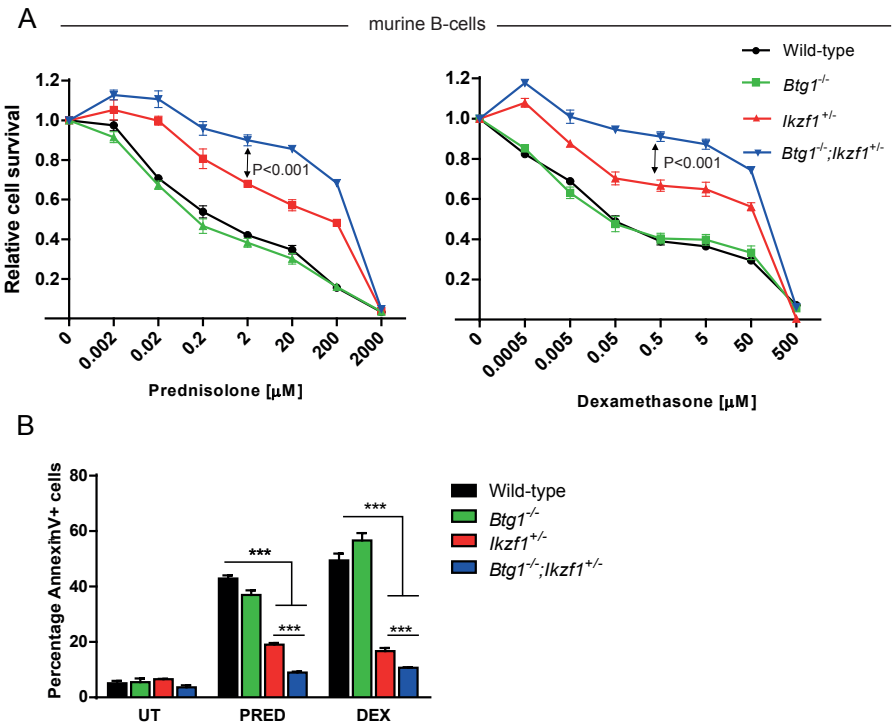

Figure 4 Glucocorticoid resistance of B cells isolated from *Btg1* knockout mice intercrossed with haplodeficient Ikzf1 animals. (A) Splenic B cells isolated from wild-type (WT), *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice were activated by lipopolysaccharide (LPS) for 48hrs and subsequently treated *in vitro* for 48 hours with increasing concentrations of prednisolone (left panel) or dexamethasone (right panel) and analyzed by MTS assay (n=6). All values were normalized to untreated (UT) B cells. Error bars represent ± standard error of the mean (SEM). *P* values were calculated based on the differences of the best-fit curve using two-sided ANOVA. **(B)** AnnexinV/7-AAD staining of WT, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ B-lymphocytes after 2 µM prednisolone or 5 µM dexamethasone treatment for 48 hours (n=4). The fraction of AnnexinV-positive cells was determined. Data represent means, and error bars represent SEM. *P* values (two-sided t test) are indicated. **P*<0.05, ***P*<0.01, and ****P*<0.001.

Discussion

BCP-ALL is a heterogeneous disease characterized by recurrent deletions enriched in specific genetic subtypes.1 For instance, it is known that deletions affecting the transcriptional co-regulator BTG1 are unevenly distributed among cytogenetic subgroups as we and others have shown that *BTG1* deletions are strongly enriched in *ETV6-RUNX1*-positive leukemia as well as *BCR-ABL1*-positive ALL. [30,31,38] The presence of these lesions in such distinct BCP-ALL subgroups may relate to the fact that deletions of *IKZF1* and *BTG1* appear to be the result of illegitimate RAG recombination, [30] as is the case for several of the other commonly deleted genes, such as *EBF1* and *PAX5*. In addition

to these earlier observations, we find a specific co-occurrence of *BTG1* and *IKZF1* gene deletions across cytogenetic subtypes, suggesting that the combined loss of *BTG1* and *IKZF1* may actively contribute to leukemogenesis. Previous studies, mostly carried out in mouse models, revealed that Ikzf1 and Arf alterations in *BCR-ABL1*-positive ALL synergize to promote the development of leukemia by conferring stem cell like properties. [39] This leads us to hypothesize that the preponderance of *BTG1;IKZF1* double-deletions in this particular subgroup may have similar consequences, although this remains to be assessed in well-established *BCR-ABL1*-positive mouse models. As *BTG1* and *IKZF1* deletions also (co)occur in lymphoid blast crises of CML, [7,31] it will be interesting to study if and how combined loss of *BTG1* and *IKZF1* drive the progression of this disease. Of all common copy number losses analyzed in our study, including *CDKN2A/B*, *PAX5*, *EBF1*, and *RB1*, only loss of *BTG1* appears to worsen outcome of *IKZF1*-deleted ALL. Our data are consistent with the findings of Moorman et al. showing that specific combinations of different deletions impact outcome in BCP-ALL. [40]

A number of different knockout mouse models have provided insight into the role of commonly deleted transcription factors in early hematopoiesis and spontaneous tumor incidence. It is evident that several of these transcriptional regulators play an important role as lymphoid specification factors and are essential for normal lymphopoiesis. [41-44] However, in the mouse, loss of these early B cell transcription factors affected in BCP-ALL, such as E2A [45] and IKZF1, [46] gives rise to T cell malignancies. E2a-deficient tumors are characterized by a strong increase in *c-Myc* mRNA expression, [45] an oncogene known to promote the development of T cell lymphomas. [47] Similarly, while *IKZF1* deletions predominantly occur in human BCP-ALL, heterozygous *Ikzf1* knockout and dominant-negative *Ikzf1* mice exclusively develop T cell malignancies, which has been attributed to activation of the Notch pathway. [36,48] In our studies we observed a lower incidence of T cell leukemia in *Ikzf1*$^{+/-}$ mice as compared to what has been reported for some other genetically engineered *Ikzf1* mouse models, where expression of dominant-negative isoforms or hypomorphic knockout alleles of *Ikzf1* yielded a higher susceptibility to T cell malignancies. [35,36,48] Similar to mice heterozygous knockout for *Ikzf1*, *Btg1* knockout mice develop T cell leukemia, while *BTG1* deletions are almost exclusively found in human BCP-ALL. [30] However, consistent with our finding that mono-allelic *BTG1* deletions are enriched in human BCP-ALL cases with *IKZF1* aberrations, *Btg1*$^{+/-}$*;Ikzf1*$^{+/-}$ mice are more prone to develop leukemia relative to *Btg1*$^{+/-}$ single-knockout mice (P=0.013). In addition, we observed a significant acceleration in the onset of disease in *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ mice as compared to *Btg1*$^{-/-}$ (P=0.026) or *Ikzf1*$^{+/-}$ mice (P=0.011), indicating that loss of these tumor suppressor genes cooperates during leukemogenesis. Genomic DNA analyses further indicate that both the wild-type *Btg1* allele and *Ikzf1* allele are maintained in the *Btg1*$^{+/-}$*;Ikzf1*$^{+/-}$ leukemias (data not shown), arguing that *Btg1* and *Ikzf1* dosage contribute to leukemia development. These data confirm that BTG1 acts as a tumor suppressor gene that cooperates with IKZF1 loss during leukemia development.

Another important finding in this study is that *BTG1* deletions define a high-risk group within the *IKZF1*-deleted subtype. Our finding that loss of BTG1 specifically enhances GC resistance mediated by Ikzf1-haplodeficiency implies that the prognostic value of *BTG1* and *IKZF1* deletions could be dependent on the upfront treatment and dose of synthetic glucocorticoids used. However, this remains to be established in future studies. The relation between *BTG1* deletions and inferior outcome was recently confirmed with the analyses of a relapsed BCP-ALL cohort, showing that *BTG1* and *NR3C1* deletions were associated with higher risk of disease progression. [49] Collectively, our data demonstrate that *BTG1* is a prognostic factor and regulator of the GC response, particularly in the context of *IKZF1*-deletions.

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Authorship Contributions

BS, JMB, RM, ET, DvIS and LvE performed experiments and collected data; BS, ET, EW, DvIS, RM, LTvdM, RPK, ES, RP, GE, MAH, NV, RS, LD, PMH, JMB, MLdB, and FNvL collected, analyzed and interpreted data; BS, JMB, MLdB and FNvL designed the research; BS, RM, ET, JMB, MLdB and FNvL wrote the manuscript. All authors reviewed and approved the manuscript.

Conflict of Interest Disclosures

The authors declare no competing financial interests.

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Supplementary Material Chapter 5

Supplementary methods

Cytogenetic and molecular analysis of patient samples

Cases were screened for high hyperdiploidy (51-65 chromosomes), *ETV6-RUNX1* and *BCR-ABL1* fusion products and rearrangements of *TCF3* and *MLL* by routine diagnostic procedures. Cases in the discovery cohort were identified as *BCR-ABL1*-like, based on a previously described expression signature of 110 probe sets. [1,2] The multiplex ligation-dependent probe amplification assay SALSA MLPA P335 ALL-IKZF1 (MRC-Holland, Amsterdam, the Netherlands) was performed according to the manufacturer's protocol and analyzed as previously described.1 A ratio lower than 0.75 for the exon intensity in patients versus the healthy control reference was considered to represent a deletion. For scoring *CDKN2A/B* deletion, it was sufficient to have either *CDKN2A* or *CDKN2B* deleted. Mutation status for *IKZF1* was neither assessed in discovery (n=533) nor validation (n=515) cohort.

Flow cytometric analysis

Murine bone marrow (BM) cells were harvested by flushing femurs with RPMI medium (Gibco//Life technologies Europe BV, Bleiswijk, The Netherlands) containing 10% FBS (Greiner Bio-One BV, Alphen a/d Rijn, The Netherlands) and 50µM □-Mercaptoethanol (Gibco). Cells from spleen and thymus were isolated through a 70µM filter using RPMI medium containing 10% FBS and 50µM □-Mercaptoethanol. Leukemic blasts isolated from either lymph nodes, spleen or thymus were analyzed by FACS using the LSRII flow cytometer (BD Biosciences, Breda, The Netherlands). Single cell suspensions were treated with red blood cell lysis buffer (Sigma-Aldrich, Zwijndrecht, The Netherlands) and pre-incubated with unlabeled anti–Fc III/II receptor (2.4G2) (BD Biosciences) to inhibit unspecific antibody binding. Cells were stained with the following antibodies purchased from BD Biosciences (Franklin Lakes, New Jersey) and eBiosciences (Vienna, Austria): anti-B220/CD45R (RA3-6B2); BP1 (6C3); CD4 (RM4-5); CD8 (53-6.7); CD11b/Mac1 (M1/70); CD24/HSA (M1/69); CD25 (PC61.5); CD43 (S7); CD44 (IM7); IgM (II/41); IgD (11-26c). The data were collected and analyzed by FlowJo V10 software (FlowJo, Ashland, Oregon).

Immunohistochemistry

Tissues isolated from diseased mice were fixed in 4% formalin for 24 hours. Specimens were embedded in paraffin, 4 µM serial sections were prepared and stained with hematoxylin and eosin (HE). For immunohistochemistry, slides were dewaxed and rehydrated prior to heat-induced antigen retrieval using sodium citrate buffer. After blocking with 2% goat serum and 2% BSA in 0.05% Tween-PBS, the slides were incubated

overnight with B220/CD45R (RM2600) (Invitrogen, Amsterdam, The Netherlands) and CD3 (A0452) (DAKO Netherlands BV, Heverlee, Belgium) antibodies. Biotinylated anti-rat IgG (BA9401) (Vector Laboratories, Peterborough, United Kingdom) and anti-rabbit IgG (BA1000) (Vector Laboratories) were used as secondary antibody and subsequently the slides were incubated with metal-enhanced diaminobenzidine in stable peroxide substrate buffer (Thermo Fisher Scientific Inc., Amsterdam, The Netherlands), counterstained with hematoxylin, and after dehydration coverslipped with Entellan. Four selected regions per murine liver or lung histological slide were used for the quantification of $CD3^+$ positively stained areas. The analysis was performed with FIJI software. [3] For the quantification of the blood smears, 10 visual fields with a 40x objective were scored for the number of blasts. In total, 10 visual fields per blood smear were averaged and for each genotype the percentage of blasts was determined for three independent mice.

VDJ rearrangement analysis

DNA was extracted from enlarged spleen or lymph nodes using the Gentra Puregene Mouse Tail kit (Qiagen, Venlo, The Netherlands). PCR analysis was performed with primers specific for the DB1-JB region of Trb1, Dβ2.1-Jβ2.7 region of Trb2, and 5'VH2-3'Cμ and 5'VH2-3'Cκ primers for the Igh and Igk genes, respectively (*Supplementary Table S1*).

Viability assays on murine splenic B cells

Spleens from healthy young (6-12 weeks of age) wild-type, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ mice were isolated and single mononuclear cells were harvested through a 70 μM cell strainer. Erythrocytes within the cell suspensions were removed by Red blood cell lysis buffer (Sigma-Aldrich, Zwijndrecht, The Netherlands). Next, splenocytes were cultured for 48 hours in RPMI 1640 medium (Life technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% penicillin/streptomycin (Invitrogen), and 50 μM β-Mercaptoethanol in the presence of 5 μg/mL lipopolysaccharide (LPS). Subsequently, splenic B-lymphocytes obtained after ficoll gradient were cultured in a 96-well plate at a density of $1x\ 10^5$ cells/well in the presence of 5 μg/mL LPS. Splenic B cells were treated with increasing concentrations of prednisolone or dexamethasone (both Centrafarm, Etten-Leur, the Netherlands). After 48 hours, relative cell viability was assessed using the CellTiter 2 96® AQueous One Solution Cell Proliferation (MTS) Assay (Promega, Madison, WI). Absorbance was acquired using a plate reader (Infinite F50; TECAN, Männedorf, Switzerland). For AnnexinV analyses, splenic B-lymphocytes obtained from young (6-12 weeks of age) wild-type, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ mice were treated for 48 hours with prednisolone or dexamethasone and stained with the AnnexinV-PE/7-AAD Viability Kit (BD Biosciences) according to manufacturer's instructions and analyzed by FACS. AnnexinV-positive cell fractions were determined by FlowJo software Version 10 (Treestar, Ashland, OR).

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Supplementary Tables

Supplementary Table S1 Oligonucleotide primers used for genotyping and IG/TR clonality analysis.

Gene locus	Forward primer (5'-3')	Reverse primer (5'-3')
Wild-type *Btg1*	CCATGCATCCCTTCTACACCC	TGCAGGCTCTGGCTGAAAGT
Knockout *Btg1*	CCATGCATCCCTTCTACACCC	CGGAGAACCTGCGTGCAATC
Wild-type *Ikzf1*	ATGGATGTCGATGAGGGTCAAGAC	AGTTCTCTGAACACAGACACACGT
Knockout *Ikzf1*	GCGGAGCTCCTCAGGTGCAGGCTGCCTATC	GACGCGTCGACGCTAACATCCTGAGGGACTGT
Trb1 (DJ)	CAGCCCCTTCAGCAAAGAT	CCTAAGTTCCTTTCCAAGACCAT
Trb2 (DJ)	GTAGGCACCTGTGGGGAAGAAACT	TGAGAGCTGTCTCCTACTATCCATT
Igh	CCCGAATTCCAGGTCCAGTTGCAGCAGWCWGG	CCCGAATTCGCTCTCGCAGGAGAC
Igk	CCCGAATTCCAGGTCCAGTTGCAGCAGWCWGG	CCCGAATTCTAACTGCTCACTGGA

Supplementary Table S2 Representation of pediatric ALL subtypes in DCOG and COALL cohorts

ALL subtypes	DCOG (N=470)		COALL (N=128)		Total (N=598)		Literature*
	N	Perc	N	Perc	N	Perc	Perc
ETV6-RUNX1	120	26%	34	27%	154	26%	25%
High hyperdiploid (51-65 chr)	95	20%	31	24%	126	21%	25%
Non-*BCR-ABL1-like* B-other	80	17%	27	21%	107	18%	10-15%
BCR-ABL1-like B-other	69	15%	23	18%	92	15%	10-15%
TCF3-rearranged	17	4%	2	2%	19	3%	5%
BCR-ABL1	22	5%	2	2%	24	4%	3%
MLL-rearranged	10	2%	1	1%	11	2%	2%
T-ALL	57	12%	8	6%	65	11%	15%

** Pui et al, Journal of Clinical Oncology 2011*

Supplementary Table S3 Co-occurrence of BTG1 deletion with other common gene deletions in BCP-ALL

	BTG1 no deletion		*BTG1* deletion		Fisher Test	
	N	Perc	N	Perc	*P*-value	Odds ratio
IKZF1 no deletion	397	0.82	31	0.65	0.0071	2.5
IKZF1 deletion	88	0.18	17	0.35		
PAX5 no deletion	352	0.73	33	0.69	0.61	1.2
PAX5 deletion	133	0.27	15	0.31		
CDKN2A/B no deletion	323	0.67	28	0.58	0.27	1.4
CDKN2A/B deletion	162	0.33	20	0.42		
EBF1 no deletion	462	0.95	39	0.81	0.0011	4.6
EBF1 deletion	23	0.05	9	0.19		
RB1 no deletion	451	0.93	40	0.83	0.042	2.6
RB1 deletion	34	0.07	8	0.17		
ETV6 no deletion	351	0.72	25	0.52	0.0046	2.4
ETV6 deletion	134	0.28	23	0.48		

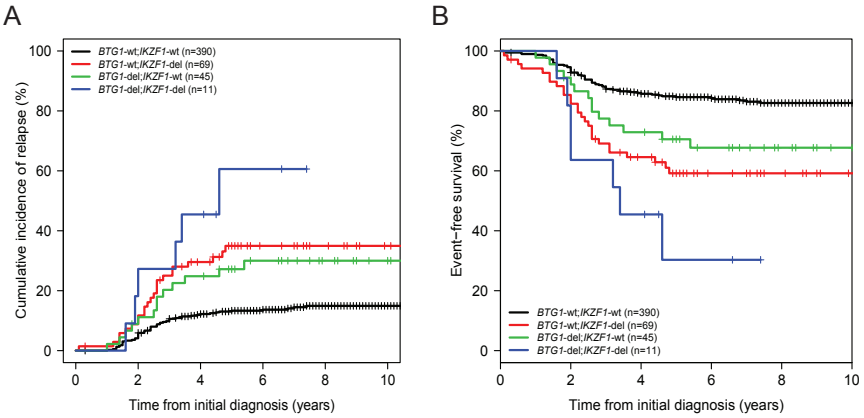
Supplementary Table S4 Outcome analysis in BCP-ALL cases double-deleted for common gene deletions and IKZF1 versus IKZF1-only deleted cases.

PAX5				**Univariate**				**multivariate**	
Deletion	**Total**	**Relapse**	**Death**	**5-yr CIR (SE)**	**Gray P**	**EFS HR (95% CI)**	**Cox P**	**EFS HR (95% CI)**	**Cox P**
PAX5;IKZF1	50	17	3	32% (7%)	0.8	1.0 (0.6-1.9)	0.9	0.7 (0.3-1.3)	0.2
IKZF1	55	18	4	33% (7%)					
PAX5	98	19	1	19% (4%)					
none	330	36	11	11% (2%)					
CDKN2A/B				**Univariate**				**multivariate**	
Deletion	**Total**	**Relapse**	**Death**	**5-yr CIR (SE)**	**Gray P**	**EFS HR (95% CI)**	**Cox P**	**EFS HR (95% CI)**	**Cox P**
CDKN2A/B; IKZF1	52	20	3	39% (7%)	0.2	1.4 (0.8-2.5)	0.3	0.9 (0.4-1.8)	0.7
IKZF1	53	15	4	26% (6%)					
CDKN2A/B	130	23	5	18% (4%)					
none	298	32	7	10% (2%)					
EBF1				**Univariate**				**multivariate**	
Deletion	**Total**	**Relapse**	**Death**	**5-yr CIR (SE)**	**Gray P**	**EFS HR (95% CI)**	**Cox P**	**EFS HR (95% CI)**	**Cox P**
EBF1;IKZF1	9	3	1	22% (15%)	0.9	1.2 (0.4-3.3)	0.7	2.1 (0.7-6.9)	0.2
IKZF1	96	32	6	33% (5%)					
EBF1	23	3	0	15% (8%)					
none	405	52	12	13% (2%)					
RB1				**Univariate**				**multivariate**	
Deletion	**Total**	**Relapse**	**Death**	**5-yr CIR (SE)**	**Gray P**	**EFS HR (95% CI)**	**Cox P**	**EFS HR (95% CI)**	**Cox P**
RB1;IKZF1	14	5	1	37% (14%)	0.7	1.2 (0.5-2.9)	0.6	0.6 (0.2-1.6)	0.3
IKZF1	91	30	6	31% (5%)					
RB1	28	5	1	18% (8%)					
none	400	50	11	12% (2%)					
ETV6				**Univariate**				**multivariate**	
Deletion	**Total**	**Relapse**	**Death**	**5-yr CIR (SE)**	**Gray P**	**EFS HR (95% CI)**	**Cox P**	**EFS HR (95% CI)**	**Cox P**
ETV6;IKZF1	25	10	0	40% (10%)	0.4	1.0 (0.5-2.0)	0.9	1.5 (0.7-3.4)	0.3
IKZF1	80	25	7	30% (5%)					
ETV6	132	11	1	8% (3%)					
none	296	44	11	15% (2%)					

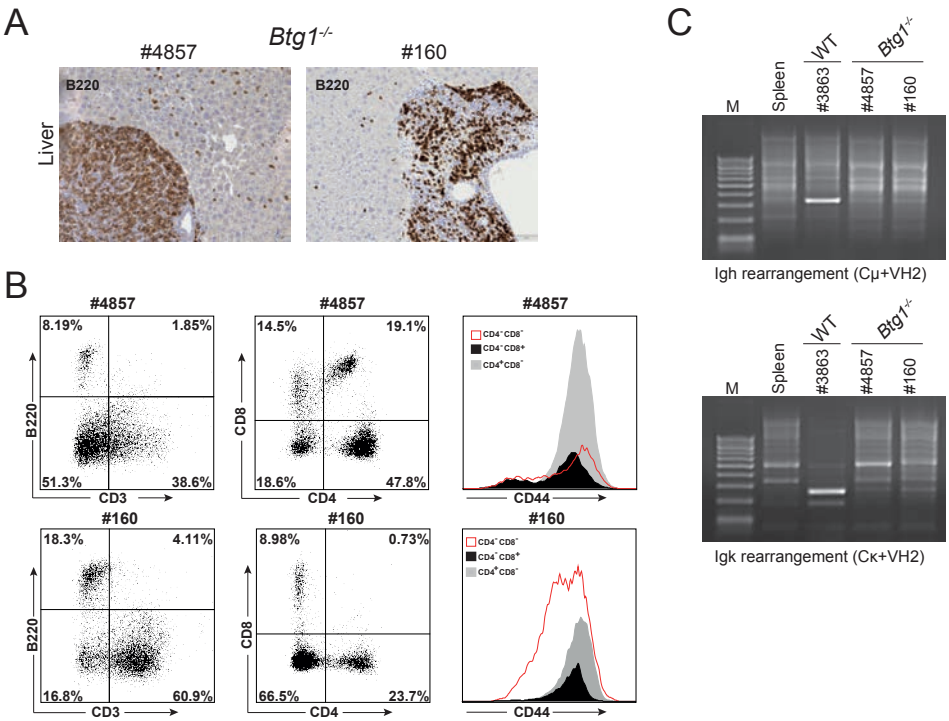
CIR, cumulative incidence of relapse; SE, standard error; EFS, event-free survival; HR, hazard ratio; CI, confidence interval; multivariate, corrected for BCP-ALL subtype and stratified for study cohort (DCOG, COALL)

Supplementary Table S5 Excel data sheet with leukemia phenotype mouse tumors. See online version at Hematologica website.

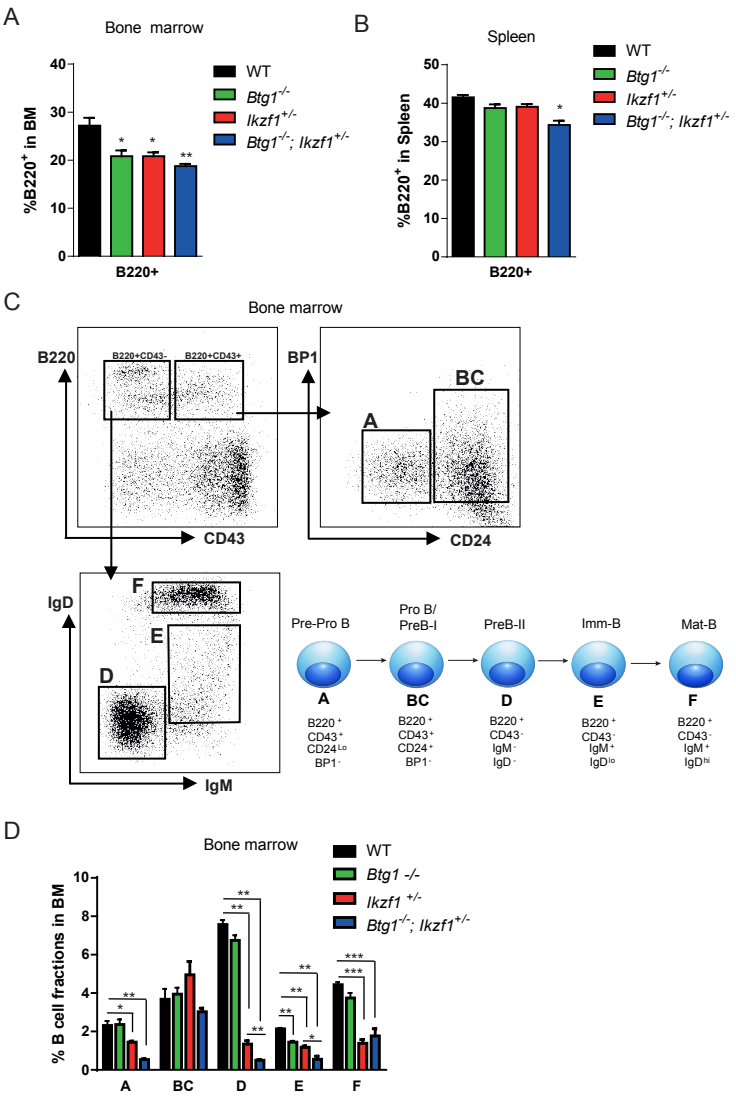
Supplementary Figures



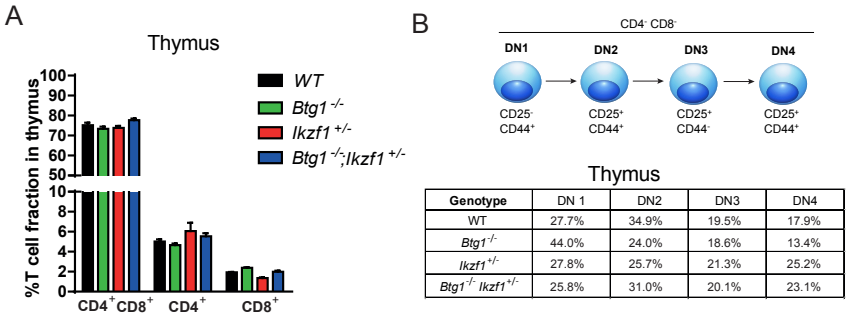
Supplementary Figure S1 Cumulative incidence of relapse (CIR) and event-free survival (EFS) curves for pediatric BCP-ALL cases from the Australian and New Zealand Children's Oncology Group (ANZCHOG) ALL8 trial with or without *IKZF1* and *BTG1* deletions. **(A)** CIR and **(B)** EFS curves for total BCP-ALL cohort (n=515). Colors: black, *IKZF1* and *BTG1*-wildtype; green, *IKZF1*-wildtype, *BTG1*-deleted; red, *IKZF1*-deleted, *BTG1*-wildtype; blue, both *BTG1* and *IKZF1*-deleted. Abbreviations: wt, wildtype; del, deletion; n, total number.



Supplementary Figure S2 Characteristics of *Btg1*-deficient T cell leukemias. (A-B) T cell leukemias in *Btg1*-deficient mice are characterized by a significant proportion of B220$^+$ B-lymphocytes within the tumor area, which can be demonstrated by B220-positive staining as determined by immunohistochemistry of infiltrated liver **(A)** and flow cytometry of the primary leukemia (B). **(B)** T cell leukemias in Btg1 knockout mice are characterized by relative high expression of CD44, which is evident in both CD3$^+$CD4$^-$CD8$^-$ fraction (red line), CD3$^+$CD4$^-$ CD8$^+$ fraction (black curve) and CD3$^+$CD4$^+$CD8$^-$ fraction (gray curve). **(C)** Immunoglobulin gene rearrangements for heavy chain (Igh) and kappa light chain (Igk) show clonal rearrangement in B cell malignancies derived from wild-type C57Bl/6J mice, but not in leukemias derived from *Btg1*$^{-/-}$ mice, indicating that B220$^+$ cells in leukemic infiltrates of *Btg1*$^{-/-}$ mice represent non-leukemic B cells.



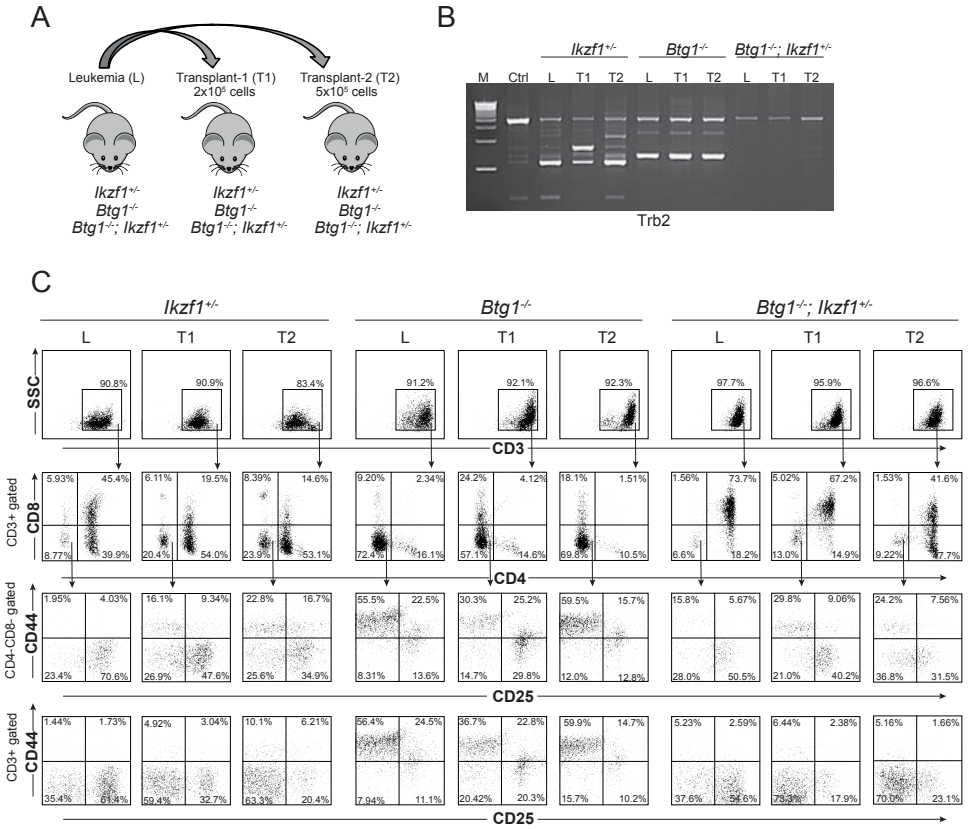
Supplementary Figure S3 Analysis of B cell development in *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice in bone marrow and spleen. Bone marrow (BM) and splenic cells from WT, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice were stained with an antibody cocktail against B220, CD43, BP-1, IgD and IgM. **(A-B)** Flow cytometric analysis on the fraction of B220^{+}-cells in bone marrow (A) and spleen (B) of the different genotypes (n=4). **(C)** Representative gating strategy of the early B-lymphoid populations in the bone marrow compartment. After gating on the viable cell population and according to B220 and CD43 expression levels, the B220^{+}CD43^{+} population was further analyzed for their BP-1 and CD24 expression to identify Hardy Fraction A (B220^{+}CD43^{+}BP-1^{-}CD24^{-}) and BC (B220^{+}CD43^{+}BP-1^{-}CD24^{+}). Hardy fraction D, E and F were identified by gating for the B220^{+}CD43^{-} population. Subsequently IgD and IgM expression levels in the B220+CD43- gate were used to distinguish Hardy fraction D (B220^{+}CD43^{-}IgM^{-}IgD^{-}) Hardy fraction E (B220+CD43-IgM+IgDlow) and F (B220^{+}CD43^{-}IgM^{+}IgD^{high}). **(D)** The relative percentages of different Hardy fractions within the bone marrow compartment is indicated for BM of WT, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice (n=4). Data for all experiments are means, and error bars represent SEM. P values (two-sided t test) are indicated. *P<0.05, **P<0.01, and ***P<0.001.



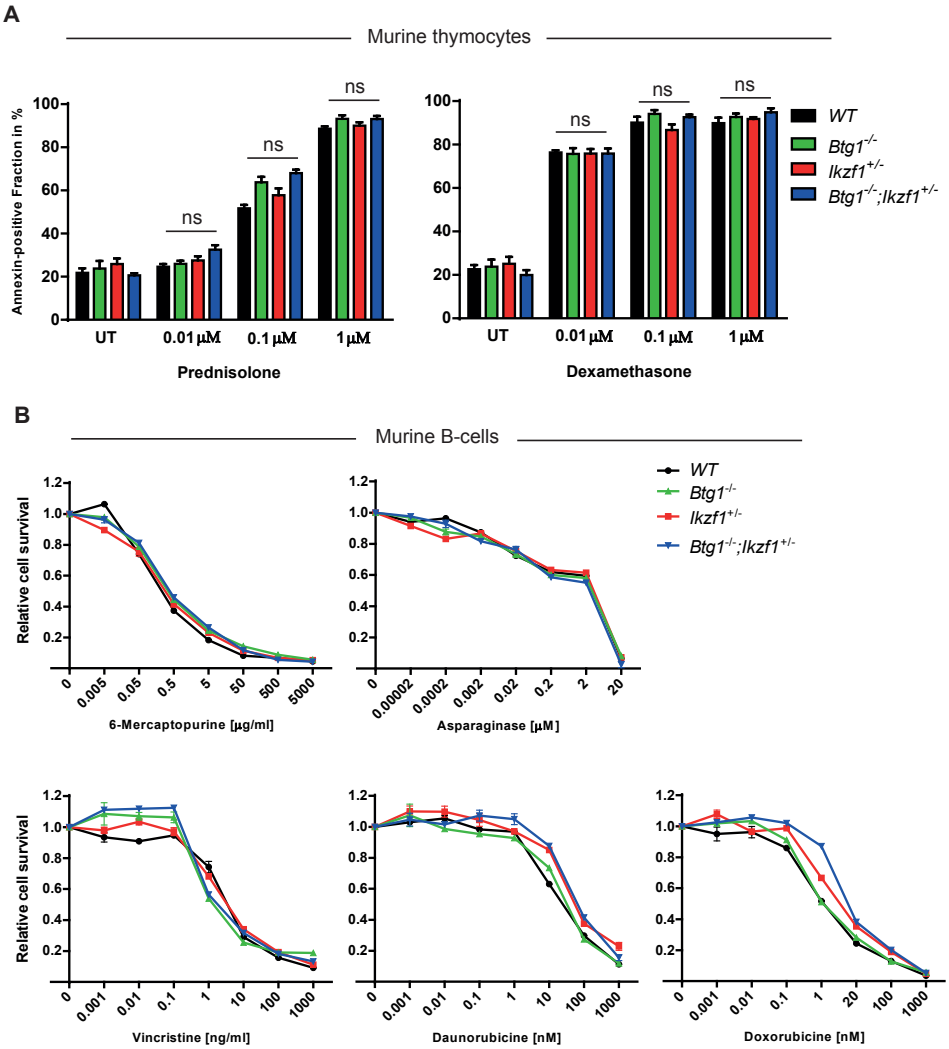
Thymus

Genotype	DN 1	DN2	DN3	DN4
WT	27.7%	34.9%	19.5%	17.9%
Btg1$^{-/-}$	44.0%	24.0%	18.6%	13.4%
Ikzf1$^{+/-}$	27.8%	25.7%	21.3%	25.2%
Btg1$^{-/-}$ *Ikzf1*$^{+/-}$	25.8%	31.0%	20.1%	23.1%

Supplementary Figure S4 Analysis of T-lymphopoiesis in *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice. **(A-B)** Flow cytometric analysis was performed on the lymphoid cells isolated from thymus of wild-type (WT), *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice (n=3). Cells were stained with an antibody cocktail against CD3, CD4, CD8, CD44 and CD25 to identify the different stages of T cell development. **(A)** Thymocytes were analyzed for expression of CD4 and CD8. **(B)** Early T cell development can be identified in the CD4^{-}CD8^{-} double negative (DN) T cell fraction. To further distinguish the DN stages of T cell development, the CD4-CD8- cells were gated for CD25 and CD44 expression. DN1 is defined as CD44^{+}CD25^{-} fraction, DN2 as CD44^{+}CD25^{+} fraction, DN3 as CD44^{-}CD25^{+} fraction and DN4 as CD44^{-}CD25^{-} fraction in the thymus. Each value represents the mean of three mice. Data are means, and error bars represent SEM.



Supplementary Figure S5 Leukemias arising in Btg1 and *Ikzf1* knockout animals are (oligo) clonal and transplantable into syngeneic mice. (A) Schematic overview of the transplantation experiments. Leukemias (L) derived from either *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$, *and* *Ikzf1*$^{+/-}$;*Btg1*$^{-/-}$ mice were transplanted intravenously into syngeneic mice at a concentration of 2x105 cells (transplant-1; T1) or 5x105 cells (transplant-2; T2). T cell leukemias were observed 4 weeks after transplantation. (B) T cell receptor beta 2 (Trb2) gene rearrangements were determined by PCR on DNA isolated from non-lymphoid tissue (earclip) as control for germline configuration, and DNA derived from L, T1 and T2 tumor tissues of *kzf1*$^{+/-}$, *Btg1*$^{-/-}$, *and Ikzf1*$^{+/-}$;*Btg1*$^{-/-}$ mice. (C) Flow cytometry was performed on the original leukemia (L) and two independent serial transplantations (T1 and T2). The upper panel shows the side scatter (SSC) and CD3 profile. The CD3-positive fraction was subsequent analyzed for CD4 and CD8 staining (second panel) and gated for the $CD4^{-}CD8^{-}$ fraction to analyze CD44 and CD25 expression (third panel) as well as on the complete $CD3^{+}$ fraction (fourth panel).



Supplementary Figure S6 Chemotherapy response in primary lymphoid cells isolated from *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice. (A) AnnexinV/7-AAD staining of WT, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ murine thymocytes after treatment with increasing concentrations of prednisolone (left panel) or dexamethasone (right panel) for 12 hours (n=4) or left untreated (UT). The fraction of AnnexinV-positive cells was determined. Data are means, and error bars represent SEM. Differences were statistically non-significant (ns) as assessed by two-sided t-test. **(B)** Splenocytes isolated from wild-type (WT), *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice were stimulated with lipopolysaccharide (LPS) for 48 hrs. Ficoll-purified splenic B cells were treated with increasing concentrations of common single chemotherapeutic agents used in ALL treatment. Cells were treated with 6-mercaptopurine, daunorubicine, vincristine or doxorubicine for 48 hours, or for 72 hours with asparaginase, and analyzed by MTS assay. All values were normalized to untreated B cells. Error bars represent ± standard error of the mean(SEM) and a two-sided ANOVA was performed for statistical analysis (n=2).

Chapter 6

Loss of IKZF1 confers resistance to pyrimidine analogs in B cell precursor acute lymphoblastic leukemia

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Manuscript in preparation

Abstract

Deletions and mutations affecting lymphoid transcription factor *IKZF1* occur in about 10-15% of patients with B cell precursor acute lymphoblastic leukemia (BCP-ALL) and predict a poor outcome. We have recently shown that loss of IKZF1 function compromises the therapeutic efficacy of glucocorticoids (GCs) in the treatment of *IKZF1*-deleted BCP-ALL. Here, we investigated whether loss of IKZF1 function also affects therapy resistance to other chemotherapeutic agents used in the treatment of ALL. We observed that CRISPR/CAS9-mediated knock-out of *IKZF1* (*IKZF1*^{KO}) in Nalm6 pre-B ALL was sufficient to make these cells highly resistant to the pyrimidine analogs gemcitabine and cytarabine (AraC) relative to *IKZF1* wild type cells. The same drug resistance profile was observed upon short hairpin RNA (shRNA)-mediated knockdown of *IKZF1* as well as in B cells isolated from *Ikzf1*$^{+/-}$ mice. We confirmed these findings by *ex vivo* profiling of patient-derived xenografts, where we observed that *IKZF1*-deleted cases were significantly ($^{10}\log IC_{50}$ 3.2 nM) more resistant to AraC and gemcitabine than those wild type for IKZF1 ($^{10}\log IC_{50}$ 1.8 nM) Previous studies have identified the metabolic enzyme cytidine deaminase (CDA) as a determinant of AraC therapy response, both in ALL and acute myeloid leukemia (AML). Consistent with these findings, we observed that loss of *IKZF1* increases the expression of *CDA* in BCP-ALL cell lines as well as mouse primary B cells. In addition, treatment of *IKZF1*^{KO} cells using pharmacological inhibitors of CDA, restored pyrimidine analog sensitivity to control levels. Together, these results demonstrate that loss of *IKZF1* confers resistance to AraC and gemcitabine, both in experimental models and in patient derived leukemic cells. Moreover, we postulate that replacing AraC with alternative nucleoside analogs may enhance treatment efficacy in this high-risk patient group.

Introduction

Over the last decades, the treatment outcome of children with pediatric B cell precursor acute lymphoblastic leukemia (BCP-ALL) has drastically improved due to improvements in ALL risk stratification and treatment regimens [1]. Despite this, still 15% of these patients develop a relapse, which strongly reduces the chance of cure. Therefore, better upfront therapies are necessary for children who are at risk of developing a relapse, while more effective and less toxic treatments for relapsed patients are urgently needed. In the last decades, the immunophenotype of the disease (B or T cell leukemia) [2], age at initial diagnosis [3] and response on treatment defined by day 8 prednisone response [4] and minimal residual disease (MRD) status [5-7] have been established as important parameters for risk stratification of ALL patients receiving current, multi-agent chemotherapy [8].

In addition, treatment response is partly determined by the (cyto)genetic context of the leukemia, which has led to the implementation of risk-adapted therapies based on specific genetic abnormalities [9,10]. By focusing on the molecular mechanisms that contribute to therapy resistance in distinct high-risk ALL subtypes, more personalized treatment strategies, aimed at preventing relapse, can be developed.

Detailed characterization of the ALL genome by SNP array profiling and next-generation sequencing (NGS) has identified novel genetic aberrations that affect response to anti-cancer agents. Deletions or mutations affecting the lymphoid transcription factor IKZF1 can be detected in approximately 10-15% of pediatric BCP-ALL patients [11,12] and several studies have identified loss of *IKZF1* as an independent poor prognostic factor in BCP-ALL [13-15]. Therefore, deletion of *IKZF1* is now considered a high-risk feature that requires an intensified treatment, but it remains to be established whether treatment intensification improves overall survival in this patient group. Over the past few years, much has been learned about the tumor suppressive role of IKZF1 during leukemia development and the molecular pathways that relate to its impact on treatment outcome. For instance, it was shown in BCR-ABL1 positive ALL that loss of *IKZF1* promotes integrin-dependent survival signaling through activation of focal adhesion kinase (FAK) [16]. Moreover, FAK inhibition potentiated the responsiveness to ABL inhibitor dasatinib in a xenograft model [17]. In addition, we recently established that single copy loss of *IKZF1* contributes to a poor response to synthetic glucocorticoids, both in genetic models and in patients [18]. However, to what extent loss of IKZF1 function affects cellular responses to other leukemia drugs has not been explored.

It is well known that loss of p53 function, activation of c-MYC, BCR-ABL1 or mutation of RAS, profoundly affect cancer metabolism, and, as a consequence, the way tumor cells respond to treatment [19,20]. A similar principle may apply to loss of IKZF1 function in ALL, as it was recently shown that B cell transcription factors, including IKZF1, function as metabolic gate-keepers, controlling glucose and energy metabolism during pre-B cell expansion [21]. We therefore investigated whether the metabolic changes associated with

loss of IKZF1 function impact the cellular responses to specific antimetabolites, such as nucleoside analogs, used in the treatment of children with ALL. Nucleoside analogs play a prominent role in the treatment of children with hematological malignancies, affecting both mitotic and quiescent cells. Once modified into nucleotides, they are incorporated into the DNA/RNA to inhibit DNA/RNA synthesis, leading to DNA fragmentation or transcriptional arrest, resulting in cell death [22]. Resistance to nucleoside analogs can be a consequence of ineffective cellular uptake, for instance due to reduced expression of nucleoside transporters, or changes in metabolic handling of these drugs [22]. For example, previous studies have identified the metabolic enzymes cytidine deaminase (CDA) and deoxycytidine kinases as key determinants of cytarabine (AraC) therapy response both in ALL and acute myeloid leukemia (AML) [22,23], while activating mutations in the 5' nucleotidase NT5C2 lead to resistance to thiopurines such as 6-mercaptopurine (6MP) and 6-thioguanine (6TG) [24,25]. In this study, we found that loss of IKZF1 function drives resistance to pyrimidine analogs, both in our experimental cell model-based drug screens as well as in patient-derived xenografts. Moreover, we established that this effect is a consequence of increased expression of metabolic enzyme CDA in response to IKZF1 loss of function.

Methods

Cell culture and cell lines

HEK293-FT cells and BCP-ALL cell lines, RS4;11 and Nalm6, were cultured in DMEM GlutaMAX medium (Life Technologies) or RPMI 1640 GlutaMAX medium (Life Technologies), respectively. Culture media were supplemented with 10% heat inactivated fetal calf serum (HI-FCS) and 1% Penicillin/Streptomycin (P/S) (Invitrogen). Cells were maintained in a humidified incubator at 37 °C with 5% CO2.

Lentiviral production and transduction

Virus particles were produced by transient transfection of HEK293-FT cells with a combination of pLKO.1, ps-PAX2 and VSV-G using Lipofectamine 2000 (Invitrogen). After 48 hours, viral supernatant was harvested, filtered, concentrated and leukemia cell lines were transduced in the presence of 1µg/mL polybrene via spin inoculation and 24 hours after transduction selected with 1µg/mL puromycin. The guide RNA (gRNA) for IKZF1 exon 3 (Forward: CACCGCTCCAAGAGTGACAGAGTCGT,Reverse: AAACACGACTCTGTCACTCTTGGAGC) was cloned into the lenti-viral vector pLKO.1 containing CAS9. CRISPR activity was confirmed via Surveyor® Mutation Detection in cell pools. Subsequently, IKZF1-gRNA and control non-targeting (NT)-gRNA (Forward: CACCGGTAGCGAACGTGTCCGGCGT, Reverse: AAACACGCCGGACACGTTCGCTACC) transfected cells were single cell cloned and screened for IKZF1 expression via western blotting and sequencing. The generation of IKZF1-shRNA and control non-targeting NT-shRNA cell lines has been described previously [18].

Primary patient samples

Bone marrow patient samples were obtained from the Dutch Childhood Oncology group (DCOG). In accordance with the Declaration of Helsinki, written informed consent was obtained from parents or legal guardians, and institutional review boards approved the use of excess diagnostic material for research purposes.

Automated drug screen

In an automated drug screen approach, we challenged *IKZF1*^{KO} cells and Nalm6 Non-targeting (NT) controls for 72 hours with a library of drugs containing 88 different compounds that are already used, or under evaluation for the treatment of pediatric cancers. Viability was assessed using CCK-8 colorimetric cell viability assay. Details of the automated drug screen procedure were described previously [26,27].

Cell viabilty assays

AraC, Gemcytabine, Cladribine, Clofarabine, Fludarabine, Methotrexate, 6-MP, 6-TG (all Centrafarm, Etten-Leur, the Netherlands) Tetrahydrouridine (THU, Abcam) and Zebularine (ZEB, Sigma) were dissolved according to the manufacturer´s guidelines. To measure therapy response in the model cell lines, a number of different apoptotic assays was used. BCP-ALL cell lines were cultured for 72 hours in the absence or presence of the chemotherapeutic agents. After this, viability was measured by amine staining, MTS and PARP Cleavage. Leukemic cells were analyzed by Fluorescence Activated Cell Sorting (FACS) using an LSRII flow cytometer (BD Biosciences, Breda, The Netherlands). Cell membrane integrity as a measure of cell death following exposure to antimetabolites was measured by amine staining with the LIVE/DEAD™ Fixable Dead Cell Stain Sampler Kit (Thermo-Fischer, L34960). This was performed according to the manufacturers protocol and assayed by flowcytometry. For co-culture experiments, MSCs were gated out via FSC/SSC gating. A representative gating strategy can be found in the supplement. The data were collected and analyzed by FlowJo V10 software (FlowJo, Ashland, Oregon).

Mitochondrial activity as a measure for viability was determined using an MTS assay. For this, cells were seeded at a density of 1.0*105 in a flat bottom 96-wells plate. Cell were treated with a broad variety of single agent chemotherapies in an increasing concentration and incubated for 72 hours. After incubation, 20 µL Cell Titer Aqueous ONE Solution (Promega) was added to each well and incubated for 3 hours and cell viability was determined via measuring the absorbance at 492 nm. Absorbance data was normalized to the untreated sample for each cell type.

Mouse studies and generation of patient-derived xenografts

Generation of C57BL/6J *Ikzf1*$^{+/-}$ mice and experimental procedure to obtain B cells has been previously described [18,28]. For patient-derived xenograft (PDX) models, NOD. *Cg-Prkdcs*^{cid}*Il2rg*^{tm1Wjl}*/Sz*J mice (NSG-mice) were used. Mice were maintained under specific pathogen-free conditions at our Central Animal Laboratory facility. All animal experiments were approved by the Animal Experimental Committee of the Radboud university medical center and were performed in accordance with institutional and national guidelines. For the expansion of primary patient cells, non-conditioned NSG mice were injected intrafemurally with 0.5-1x10^6 viable cells. After 2-4 months these mice developed leukemia with an immunophenotype and genetic composition that in the large majority of cases is identical to the original material at the time of injection. Leukemic cells were harvested by flushing femurs with RPMI medium containing 10% FBS and 50μM β-Mercaptoethanol (Gibco). Leukemic blasts from spleen were isolated through a 70μM filter using RPMI medium containing 10% FBS and 50μM β-Mercaptoethanol. The ex vivo co-culture has been described previously [26]. In short, hTERT immortalized mesenchymal stem cells (MSCs) [29,30] were seeded in a 96-wells format and allowed to settle for 24 hours prior to the addition of ALL xenografts. ALL cells were allowed to settle for 24 hours before chemotherapeutic agents were added in increasing concentrations. After 3 days of incubation, cell death was analyzed by Amine staining using flow cytometry (Supplemental Figure 1).

Quantitative RT-PCR

RNA was extracted using a RNeasy minikit (Qiagen) according to the manufacturer´s protocol. This was followed by Superscript cDNA synthesis (*Bio-Rad*, *Hercules*, *CA*), according to manufacturer´s protocols. CDA mRNA expression levels in NALM6, RS4;11 and murine splenocytes were determined by quantitative PCR reactions using *Power SYBR® Green PCR master mix* (Applied Biosystems, Carlsbad, CA) in combination with mouse specific (Forward:ATGGCACATTCGTAGTCAGG Reverse: CTTCCAAGAGCCGTTCCTTAG) or human specific (Forward: CCGGATGGTACGTATATTGTCATG, Reverse: TTATGAAGTTCTCCAGGTGGC) CDA primers in *the CFX96 TouchTM Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA)*. Mouse and human CDA mRNA expression levels were normalized to mouse HPRT (Forward: TCGGTCGGAGATGATCTCTCAAC, Reverse: AGAGGTCCTTTTCACCAGCA) and human HPRT (Forward: GGTCCTTTTCACCAGCAAGCT, Reverse: TGACACTGGCAAAACAATGCA) respectively. *HPRT* mRNA expression was used as a reference to obtain the relative fold expression of target genes using the comparative cycle threshold $2^{(-\Delta\Delta Ct)}$ method.

Western blot analysis

Leukemic cells were lysed in laemmli protein sample buffer and treated with benzonuclease for 30 minutes, prior to boiling. Proteins were isolated to determine the extent of PARP cleavage as a measure of apoptosis and for expression levels of relevant proteins. Purified proteins were separated by SDS-PAGE and then transferred to PVDF membranes (Amersham Biosciences). After protein transfer, membranes were blocked according to the specific antibody and stained with primary antibodies for PARP (9542, Cell signaling), IKZF1 (AF4984, R&D systems) and □-Tubulin (T6557, Sigma). After incubation, membranes were washed in PBS-0.02% Tween, followed by horseradish peroxidase (HRP) conjugated secondary antibody. Proteins were visualized with ECL reagent (GE Healthcare) and expression was detected with Fluorchem (Cell biosciences, Santa Clara, USA). Quantification was performed with AlphaView software version 3.3.10 (Cell biosciences, Santa Clara, USA).

CDA activity measurements

CDA activity in cell lysates was determined by photometric detection of the ammonia release after reaction with Nessler reagent (Sigma-Aldrich). In brief, $50x10^6$ viable cells were lysed by shear stress in buffer consisting of 0.07 M potassium dihydrogenphosphate and 0.07 M disodium hydrogenphosphate (adjusted to pH 7.5) [31]. Lysates were centrifuged for 5 min at maximum speed (14.000 rpm). Subsequently 20 µL supernatant was incubated with 80 µL 4 µM cytidine with or without 100 µM Tetrahydrouridine (THU, Abcam) at 37°C for 16 hours. The reaction was stopped by the addition of 62,5 µL of trichloroacetic acid (24.5% [w/v]; Sigma-Aldrich). After centrifugation, 15 µL of the supernatant was added to 120 µL of Nessler solution diluted with deionized water (1:8). The optical density of the solution was measured at 450 nm using the Multiskan Ascent plate reader (MTX Lab Systems).

Statistical analyses

Statistical analyses for cell viability assays and quantitative real-time polymerase chain reaction (qRT-PCR) were performed using PRISM6 (GraphPad Software, La Jolla, CA). For MTS assays, two-sided ANOVA was performed to assess differences between the best-fit curves. For the Nessler Reaction, Amine staining, PARP cleavage and qRT-PCR, a student's t test was performed. For the *ex vivo* patient-derived xenograft data (Live/dead staining), the statistical significance in the *IKZF1*–deleted group versus *IKZF1*-WT group was calculated using the best fit area under the curve method using PRISM6 (GraphPad Software, La Jolla, CA). All statistical tests were performed two-sided and *P* values of less than 0.05 were considered statistically significant.

Results

Loss of IKZF1 drives resistance to pyrimidine analogs cytarabine and gemcitabine in BCP-ALL cell lines

We and others have recently shown that *IKZF1* alterations contribute to therapy resistance to leukemic cells via distinct mechanisms, including enhanced cell adhesion and modulation of glucocorticoid response [16,18]. However, the effect of *IKZF1* aberrations on the response to other drugs normally used in ALL treatment has not been investigated. Recent studies suggest that B cell developmental genes such as *IKZF1* are capable of altering the metabolic state of expanding leukemic cells [21], which in principle, could affect response to antimetabolites. While antimetabolites are closely resembling the chemical structure of their natural counterparts, these chemical analogs incorporate into the DNA during DNA repair and replication to induce apoptosis. Here, we investigated whether loss of IKZF1 function affects sensitivity to specific chemotherapeutic drugs using Nalm6 pre-B ALL cells that were either wild type for *IKZF1* or carried a heterozygous loss of function allele (*IKZF1*^{KO}), obtained by CRISPR/CAS9-mediated targeting of the IKZF1 locus. Western blot analysis confirmed successful reduced expression of the target protein (Figure 1A). In an automated drug screen, we challenged *IKZF1*^{KO} cells and NT control cells with a library of drugs containing 88 different compounds that are already used, or under evaluation for the treatment of pediatric cancers and measured viability via an CCK-8 colorimetric cell viability assay [26] (Supplementary Table 1). We observed that for the pyrimidine analogs gemcitabine and cytarabine (AraC) the IC_{50} concentration of *IKZF1*^{KO} cells was about 1000-fold increased, compared to control cells (Figure 1B). To confirm the observed resistance phenotype by independent cell viability assays, we determined accessible amine as indicator of cell membrane integrity (Figure 1C) or assessed sub-G1-phase with a cell cycle profile (Figure 1D) as direct measurement of apoptosis with flow cytometry. Both assays confirmed that *IKZF1* ^{KO} cells were significantly more resistant to AraC and gemcitabine treatment. In line with these observations, we found that shRNA-mediated knockdown of IKZF1 in Nalm6 and RS4;11 led to pyrimidine analog resistance (Supplemental Figure 2), while a similar phenotype was observed in mouse *Ikzf1*-haplodeficient B cells (Supplemental Figure 3).

To validate the observed resistance to pyrimidine analogs in a model more closely resembling ALL in the patient, we exposed primary leukemic cells expanded as patient-derived xenograft (PDX) models to AraC using an ex vivo MSC co-culture system [26,32] (Figure 1E). Consistent with our previous findings in BCP-ALL cell lines, samples obtained from *IKZF1*-deleted tumors (n=5) were significantly more resistant to AraC (^{10}logIC_{50} 3.2 nM), relative to xenografts wild type for *IKZF1* (*IKZF1*-WT; n=5) (^{10}logIC_{50} 1.8 nM) (Figure 1E/F/G). Intriguingly, while the overall response to AraC was attenuated in all *IKZF1*-deleted samples, in four out of five cases, a sub-population of leukemic cells was observed that was completely refractory to AraC (Figure 1F).

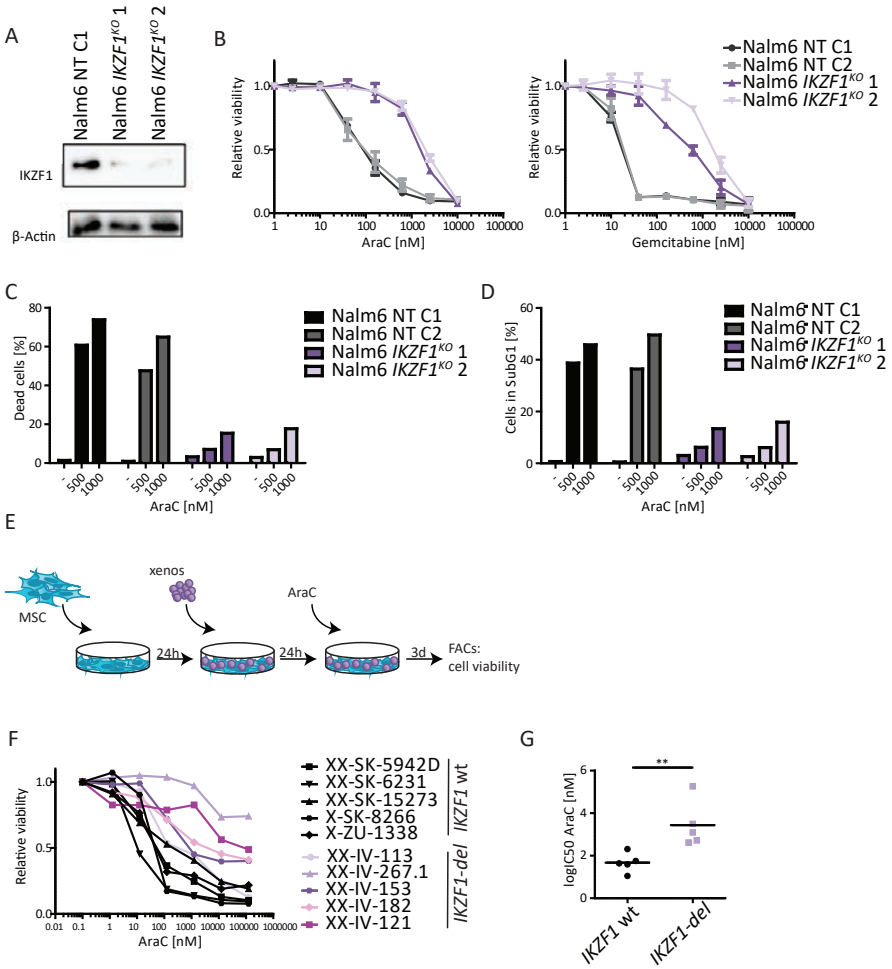

Figure 1 Loss of IKZF1 induces resistance to pyrimidine analogs in BCP-ALL cell lines and patient derived xenografts (A) A single allele of the *IKZF1* gene was disrupted by CRISPR/Cas9-mediated genome editing introducing a frame shift mutation in Nalm6 cells. Cells were subcloned and were evaluated for IKZF1 expression using Western blot. **(B)** Nalm6 control or *IKZF1*^{KO} cells were treated for 72 hours with increasing concentrations of AraC (left panel) or gemcitabine (right panel) and analyzed using an MTT based viability assay. All values were normalized to untreated Nalm6 cells. Error bars represent ± standard error of the mean (SEM). **(C)** Nalm6 control and *IKZF1*^{KO} cells were either treated with 500 or 1000nM AraC or left untreated for 72 hours before the presence of apoptotic cells was measured by flow cytometry using amine staining based on cell membrane integrity. **(D)** DNA fragmentation was assayed in the same cells by flow cytometry using Hoechst dye to detect the sub-G1 (apoptotic) fraction. **(E)** Schematic overview representing the workflow used to test drug sensitivity in PDX samples. Briefly, hTERT immortalized MSCs were seeded in a 96 wells format and allowed to settle for 24 hours prior to the addition of ALL xenografts. Cells were allowed to settle for 24 hours before AraC was added in increasing concentrations. **(F)** After 3 days of incubation, cell death was analyzed by 7AAD staining using flow cytometry. **(G)** AraC log IC50 of the graphs in F were calculated. Asterisks indicate a significant difference: ** p<0.01

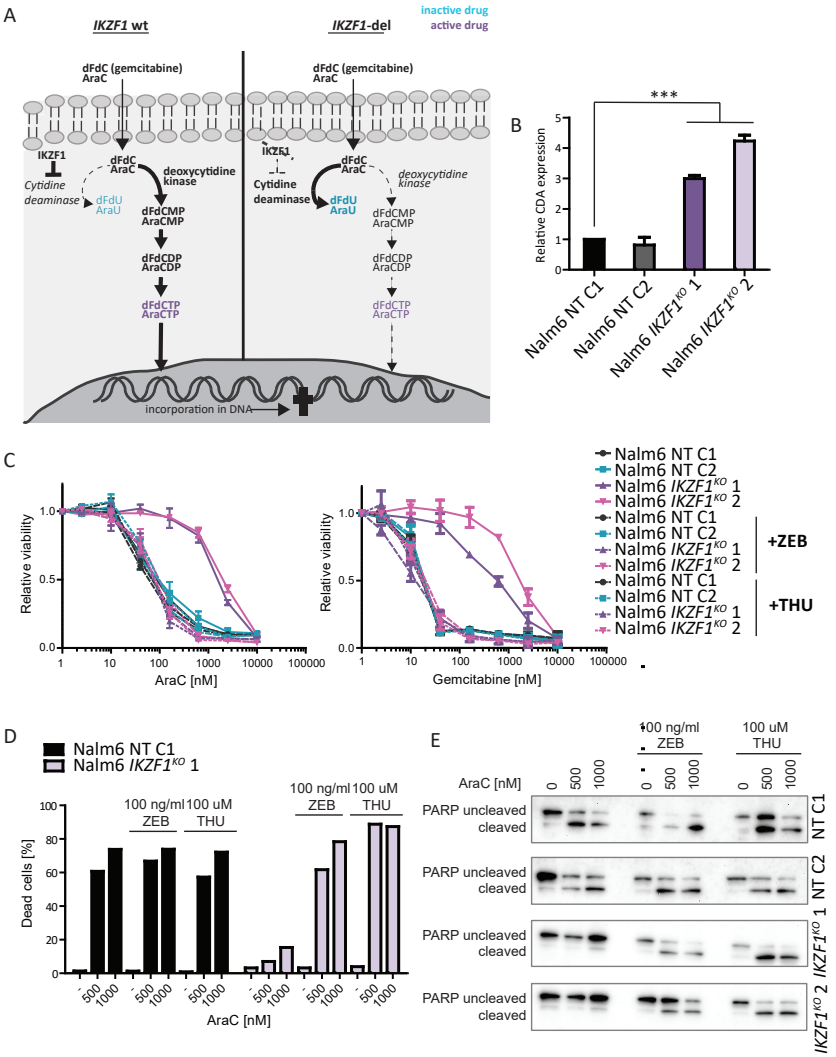

Figure 2 Inhibition of Cytidine Deaminase (CDA) in *IKZF1*^{KO} cells reverses their resistance phenotype against AraC (A) Schematic overview of of AraC and gemcitabine metabolism. **(B)** Relative mRNA expression of CDA in Nalm6 and *IKZF1*^{KO} cells as measured by real time quantitative PCR. Data are normalized for HPRT expression and shown as mean ± SD of n = 3. Statistical significance was determined by unpaired 2-sided t test. **P < 0.01. **(C)** Nalm6 NT controls and IKZF1KO cells were treated for 72 hours with increasing concentrations of AraC (left panel) or gemcitabine (right panel) in the presence or absence of the CDA inhibitor zebularine (ZEB; 100 ng) or tetrahyrouridine (THU, 100uM) and analyzed using an MTT based viability assay. **(D)** Nalm6 control and *IKZF1*^{KO} cells were either treated with 500 or 1000nM AraC in the presence or absence of ZEB or THU for 72 hours before the presence of apoptotic cells was measured by flow cytometry using amine staining (n=3) **(E)** The same cells were analyzed for caspase activity as measured by PARP cleavage using Western blot. A representative example of multiple experiments (n = 3) is shown.

Pyrimidine resistance induced by IKZF1 loss is a result of increased cytidine deaminase activity

Previous studies have identified the metabolic enzyme cytidine deaminase (CDA) as a key determinant of AraC therapy response both in ALL and AML [22,23]. CDA catalyzes the deamination of cytosine to uracil as part of the nucleotide salvage pathway [22], but it also catalyzes the conversion of AraC into AraU, leading to inactivation of the drug (Figure 2A). Using quantitative RT-PCR, we observed a 3 to 4-fold increase in CDA mRNA expression in our model cell line Nalm6 (Figure 2B). Consistent with this observation, B cells isolated from 8 to 14 weeks-old *Ikzf1*$^{+/-}$ mice showed a significant increase in CDA mRNA levels (Supplemental Figure 4).

To further investigate the relation between CDA activity in *IKZF1*^{KO} cells and AraC resistance in BCP-ALL, we exposed Nalm6 *IKZF1*^{KO} cells and controls to two independent pharmacological inhibitors of CDA, Zebularine (Zeb) or tetrahydroyuridine (THU) in the presence of AraC. For both inhibitors, concentrations were chosen that do not affect cell viability as a single agent (Supplemental Figure 5). However, in combination with AraC, both Zeb and THU restored pyrimidine analog sensitivity of *IKZF1*^{KO} cells to control levels in MTS assays (Figure 2C). We were able to validate the inhibitor-mediated reversal of AraC sensitivity by amine staining (Figure 2D). Additionally, determination of PARP cleavage indicated that pharmacological inhibition of CDA effectively re-sensitizes *IKZF1*^{KO} cells to pyrimidine analogs (Figure 2E).

To test whether CDA activity is indeed elevated in *IKZF1*^{KO} cells, we assayed enzyme activity in cells lysates from WT and *IKZF1*^{KO} Nalm6. For this, we measured the release of ammonia following the deamination of cytidine using a Nessler reaction. Compared to control lysates, those obtained from *IKZF1*^{KO} cells showed a strong increase in CDA activity as measured by the formation of amines during the Nessler reaction. In line with previous observations, treatment of *IKZF1*^{KO} lysates with the CDA inhibitor THU was sufficient to restore CDA activity to levels comparable to that of the control lysates (Figure 3). We conclude from these experiments that increased activity of CDA contributes greatly to AraC resistance in *IKZF1*-deleted BCP-ALL.

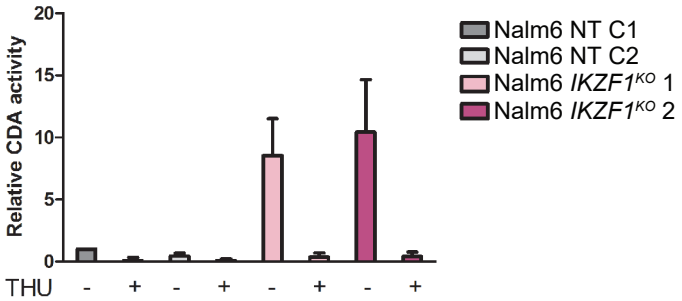

Figure 3 Loss of IKZF1 function leads to increased CDA activity. Lysates from Nalm6 NT control C1 and C2 and *IKZF1*^{KO} cells were incubated with a cytidine containing buffer for 16 hours. Ammonia release was analyzed by absorbance measurements after a Nessler reaction. The CDA inhibitor tetrahyrouridine (THU) was added to confirm contribution of CDA in this reaction. Results represent the mean and standard deviation from two independent experiments performed in duplicate.

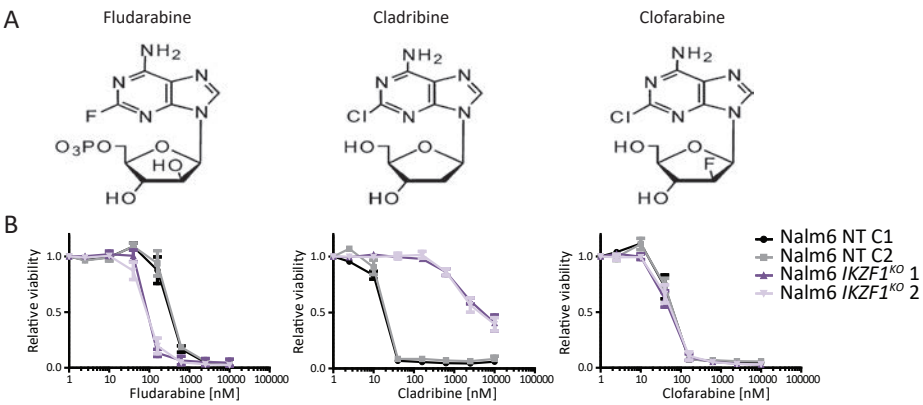

Figure 4 *IKZF1*^{KO} cells show differential sensitivity to adenine-containing antimetabolites

(A) Structural formula of three different adenine-containing antimetabolites. **(B)** Nalm6 NT controls or *IKZF1*^{KO} cells were treated for 72 hours with increasing concentrations of fludarabine, cladribine and clofarabine and analyzed using a MTT based viability assay. All values were normalized to untreated Nalm6 cells. Error bars represent ± standard error of the mean (SEM) from 3 independent experiments.

Loss of IKZF1 function affects the cellular response to other antimetabolites

In addition to pyrimidine analogs, we determined the response to three adenine-containing nucleosides, clofarabine, cladribine and fludarabine (Figure 5) in *IKZF1*^{KO} knockout cells. While no difference in cellular sensitivity was found to clofarabine between control and *IKZF1*^{KO} cells, we observed high resistance to cladribine in *IKZF1*^{KO} cells. Surprisingly, the IC_{50} to fludarabine was significantly lower for *IKZF1*^{KO} cells in comparison to control cells, where *IKZF1*^{KO} cells appeared to be about five-fold more sensitive to fludarabine. In view of these findings, it will be important to investigate whether antimetabolites other than AraC, such as fludarabine or clofarabine, may be used to enhance cellular response to therapy in *IKZF1*-deleted ALL.

Discussion

Pyrimidine analogs, such as AraC and gemcitabine, are common components of leukemia treatment regimens. Resistance to these drugs remains a problem in the effective treatment of hematopoietic malignancies, and methods to predict resistance in patients are needed. In this study, we demonstrate that loss of IKZF1 confers resistance to pyrimidine analogs both in BCP-ALL cell line model system as well as in *ex vivo* PDX of primary BCP-ALL. In addition, we show that an increase in CDA expression and activity drives this resistance in *IKZF1*-deleted BCP-ALL.

Exposing *IKZF1*^{KO} cells to two independent specific inhibitors of CDA, ZEB and THU, re-sensitized these cells to AraC and Gemcitabine. However, to further support the relation between increased CDA expression and AraC resistance in *IKZF1*^{KO} cells, shRNA-mediated knockdown of CDA in *IKZF1*^{KO} cells should be performed. In addition, CRISPR-CAS9 mediated knockout of *CDA* in BCP-ALL cell lines will further shed light on the direct effect of CDA in drug sensitivity in BCP-ALL independent of *IKZF1* aberrations. To further support the observation that loss of IKZF1 function enhances the activity of CDA, lysates from wild-type and *IKZF1*-deleted primary patient samples should also be measured in a deamination assay to further demonstrate that AraC resistance is a direct consequence of enhanced CDA activity

Although our results would suggest that pharmacological inhibition of CDA could be of potential use *in vivo*, CDA activity in the liver or other organs protects these cells from AraC induced toxicities during treatment. Thus, strategies aimed at systemically reducing CDA activity will likely result in severe toxicities. Therefore, identification and targeting of hematopoietic-cell specific regulators of CDA may be used to selectively enhance sensitivity to AraC in leukemic cells in the future. In contrast to the findings in our model cell lines, we did not observe an effect of the pharmacological inhibitors Zeb and THU on AraC sensitivity in our ex vivo co-culture experiments (data not shown). We attribute this

phenomenon to the MSC feeding layer, which is described to function as a "sink" for certain drugs and inhibitors, in which the MSC feeding layer metabolizes drugs before they can affect the supported leukemic cells [33]. In addition, the impact of deoxycytidine kinase as functional opponent of CDA should be studied to fully understand how AraC is metabolized in *IKZF1*^{KO} cells [34]. The differential sensitivity to AraC, but not other antimetabolites, argues that not a generic apoptotic pathway, but rather a specific metabolic pathway is responsible for the observed resistance phenotype. CRISPR/CAS9 based reverse genetics screens could identify pathway components that enhance response to AraC, for instance by regulating the expression of CDA. Most ALL treatment protocols rely on combinations of nucleoside analogs including Cytarabine (AraC), 6-mercaptopurine (6MP) and other antimetabolites, such as the antifolate methotrexate (MTX) during consolidation phase [35]. This makes it difficult to assess the leukemia response to individual components of the treatment

The finding that *IKZF1*^{KO} cells show a differential response to adenine-containing nucleosides clofarabine, cladribine and fludarabine was unexpected. While no difference in cellular sensitivity was found to clofarabine between wild type or *IKZF1*^{KO} cells, *IKZF1*-deleted cells were highly resistant to cladribine. In contrast, *IKZF1*^{KO} cells were highly sensitive to fludarabine. These findings suggest that treatment of *IKZF1*-deleted tumors may benefit from a switch from cladribine to clofarabine or the addition of fludarabine to the protocol. We found that *IKZF1*-deleted PDX samples from our collection were significantly resistant to AraC, but we need to evaluate historical data and confirm the phenotype in a broader cohort to fully capture the genetic complexity of *IKZF1* deletions. Several studies indicate that the genetic context of *IKZF1* deletions is important because co-occurring genetic events in this so-called *IKZF1*^{Plus} patient group can alter the prognostic value of *IKZF1* deletions [36]. For example, we have recently established that combined loss of BTG1 and IKZF1 represents a patient group with poor outcome in ALL and is characterized by a strong GC- resistance phenotype [37]. In contrast, the presence of ERG deletions has been shown to negate the negative effect of IKZF1-deletions on outcome [38]. Future efforts should therefore include these more complex genetic cases to understand whether *IKZF1*^{Plus} cases differ in their response to pyrimidine analogs. Remarkably, our PDX models revealed that 4 out of 5 *IKZF1* deleted cases contained a completely resistant fraction even under high concentrations of AraC. We speculate that clonal differences within these PDX contribute to this phenomenon and it would be interesting to fully genetically characterize those subclonal populations selected by chemotherapy to identify drivers of resistance in those *IKZF1*-deleted PDX models. In this context, poor responses to pyrimidine analogs have been observed to be enriched in a cohort of BCR-ABL-like leukemias [39]. These "B-others" show and increased incidence of relapse, while deletions of IKZF1 are prevalent. Therefore, it would be interesting to see whether *IKZF1* deletions can also be linked to AraC resistance in this leukemia subgroup.

Although IKZF1 is primarily a regulator of lymphoid development, there is evidence supporting a role for this transcriptional regulator in myeloid differentiation. For

instance, IKZF1 is implicated in megakaryopoiesis, which involves cooperation with transcriptional regulators such as RUNX1 and GATA1 [40]. Moreover, deletions in *IKZF1* were found to occur during progression from myeloid proliferative neoplasms to AML [41]. Indeed, deletion of chromosome seven, which harbors the *IKZF1* gene, is associated with poor outcome in AML [42], although response to AraC in this AML subgroup has not been determined. We therefore hypothesize that loss of IKZF1 may contribute to AraC resistance not only in ALL but also in AML. Given the fact that AraC is a cornerstone of AML treatment and CDA activity is a key determinant of AraC responsiveness in AML, future efforts should therefore explore the relation between IKZF1 and AraC resistance and the underlying regulatory network in ALL and AML.

Conclusion

Taken together, this study highlights a role for loss of IKZF1 in resistance to pyrimidine analogs. Currently, treatment protocols are already adjusted based on *IKZF1* status, in which patients with *IKZF1*-deleted leukemia receive a higher dose of methotrexate and an extra year of consolidation therapy. Based on our findings, replacing AraC with alternative antimetabolites may be considered for this ALL subgroup. For instance, the introduction clofarabine or fludarabine in upfront protocols may prevent the outgrowth of resistant clones and consequently reduce the number of relapses in *IKZF1*-deleted ALL.

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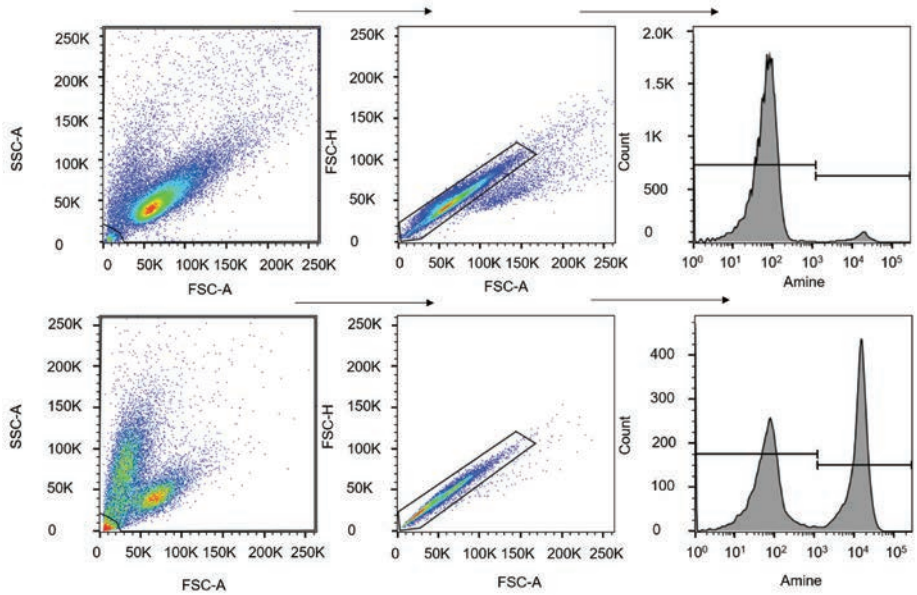
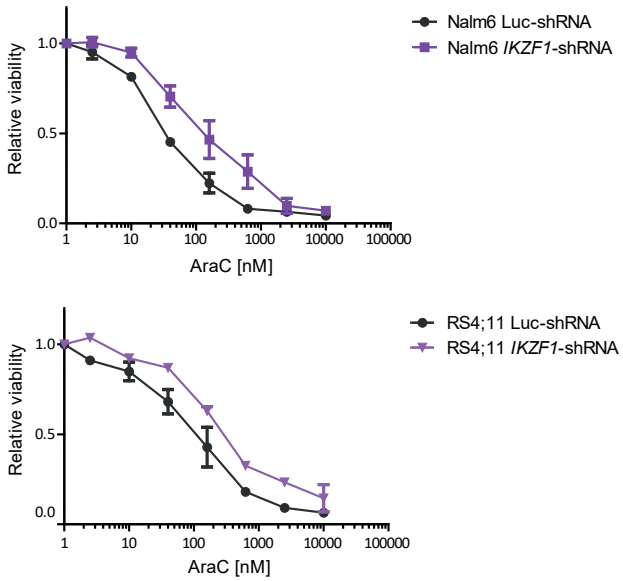
Supplemental Information Chapter 6

Supplementary Table 1 An automated drug screen was used to compare the efficacy of 88 different compounds in Nalm6 leukemic cells, either wild type for *IKZF1* or carrying a deletion affecting *IKZF1* expression or function. Indicated in this table are those drugs that elicit reduced cellular responses in *IKZF1*-deleted cells relative to controls. + indicates degree of resistance, the more +, the more resistant the *IKZF1*-deleted cells.

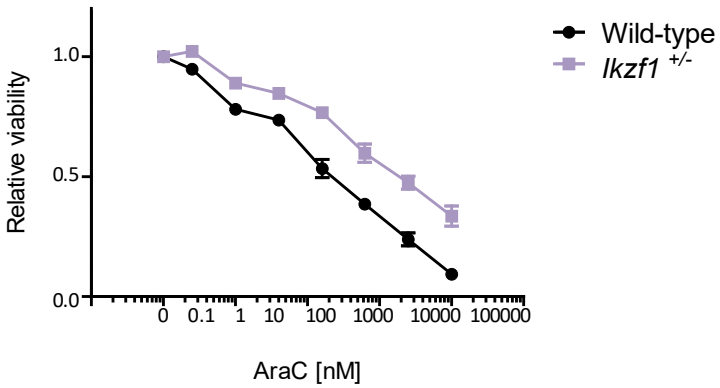
Drug	Working mechanism	Reduced cellular response in *IKZF1* deleted ALL
Nutlin	Inhibition of MDM2,p53 upregulation	+
Methotrexate	Inhibits de novo DNA synthesis	+
6-MP	inhibits purine nucleotide synthesis	-+
Obatoclax	Bcl-2 inhibitor	-+
ZM-447439	ATP-competitive inhibitor for Aurora A and Aurora B	++
XL228	inhibitor targeting IGF1R, the Aurora kinases, FGFR1-3, ABL and SRC family kinases	++
Dexamethasone	Synthetic Glucocorticoid	+
I3	Notch	+
AZD1152-HQPA	Aurora Inhibitor	-+
Velcade	proteasome inhibitor	++
AT9283	Aurora Inhibitor	+
Mitoxantrone	type II topoisomerase inhibitor, it disrupts DNA synthesis and DNA repair	++
Gemcitabine	nucleoside analog family of medication. It works by blocking the creation of new DNA	+++
Cytarabine	Interferes with DNA synthesis in S phase	++



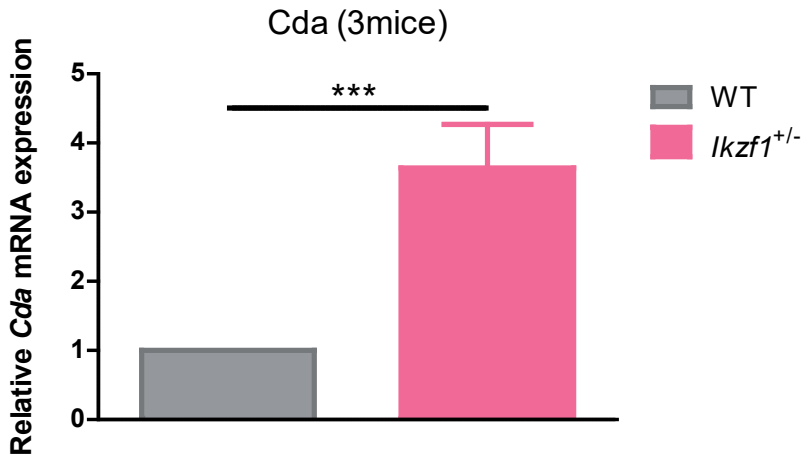
Supplementary Figure 1 Representative gating strategy of primary xenografted patient samples on an MSC co-culture. After gating out debris from the viable cell population and the exclusion of doublets, population was further analyzed by amine staining.



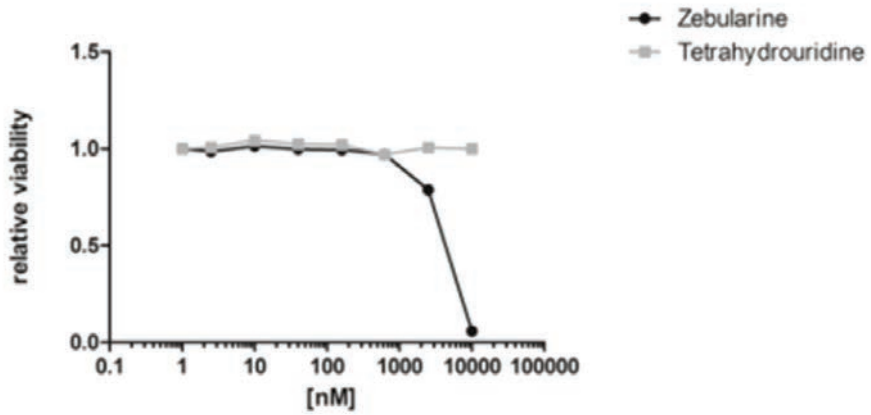
Supplemental Figure 2 Nalm6 (Upper panel) and RS4,11(Lower panel) transfected with control or IKZF1-*shRNA* were treated for 72 hours with increasing concentrations of AraC and analyzed using an MTT based viability assay. All values were normalized to untreated Nalm6 cells. Error bars represent ± standard error of the mean (SEM).



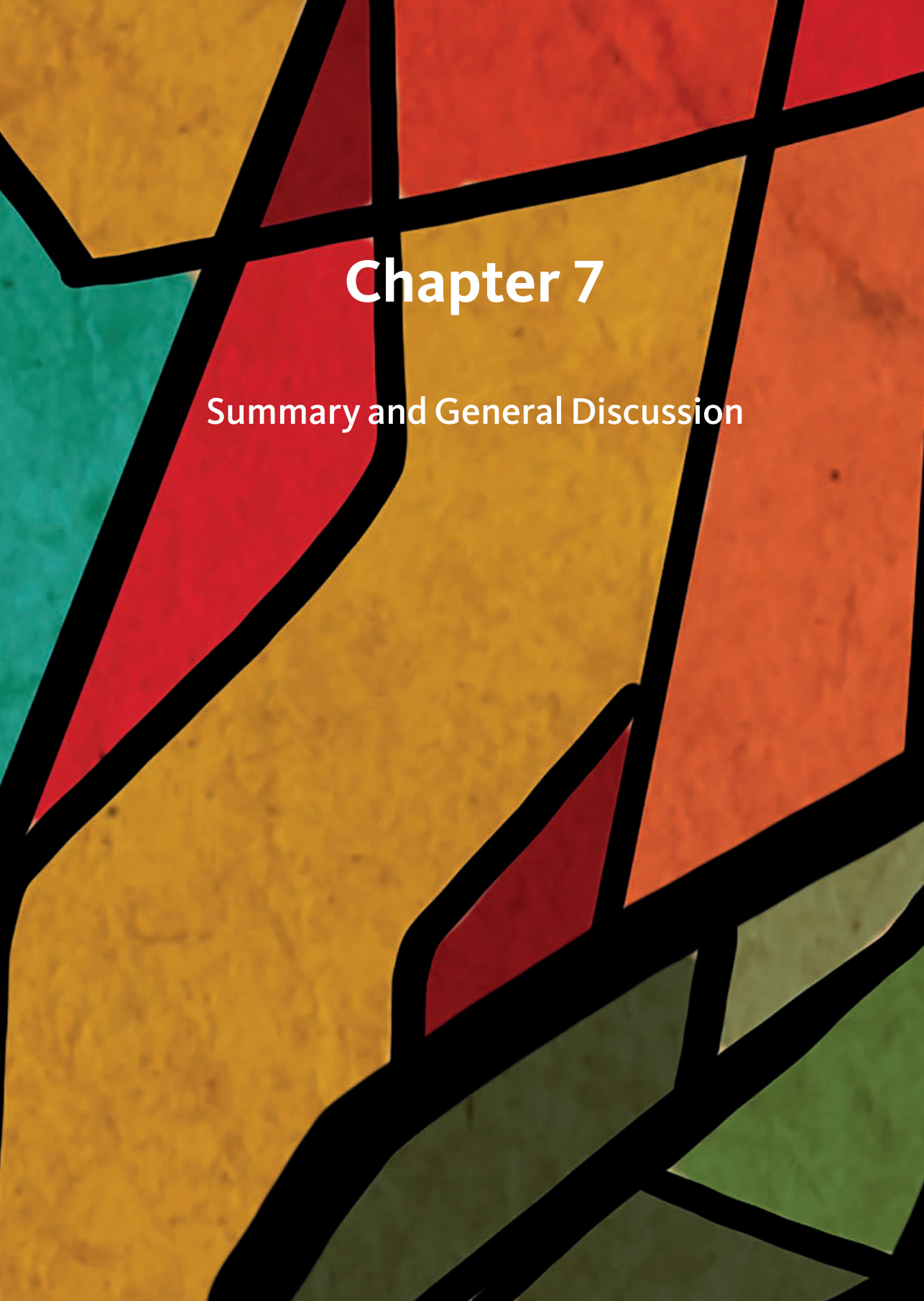
Supplemental Figure 3 LPS-activated B cells isolated from wild-type and *Ikzf1*$^{+/-}$ mice were incubated for 72 hours with increasing concentrations of AraC and analyzed using an MTT based viability assay. All values were normalized to untreated B cells. Error bars represent ± standard error of the mean (SEM).



Supplemental Figure 4 Relative mRNA expression of *Cda* in B cells isolated from wild-type and *Ikzf1*$^{+/-}$ mice as measured by real time quantitative PCR. Data are normalized for HPRT expression and shown as mean ± SD of n = 3. Statistical significance was determined by unpaired 2-sided t test. $^{**}P < 0.01$.



Supplemental Figure 5 Nalm6 were incubated for 72 hours with increasing concentrations of either Zebularine or Tetrahydrouridine and analyzed using an MTT based viability assay. All values were normalized to untreated Nalm6 cells. Error bars represent ± standard error of the mean (SEM).



Chapter 7

Summary and General Discussion

Summary

B cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common malignancy in children. During the last decades, improvements in risk stratification and treatment protocols have led to cure rates of almost 90% in BCP-ALL. However, still approximately 10 % of all BCP-ALL patients relapse after initial treatment success, often due to chemotherapy resistance. To understand why these leukemic cells are resistant to chemotherapy, we have to acknowledge that tumors are characterized by a broad variety of cytogenetic aberrations. Many of these are correlated with the survival outcome of BCP-ALL patients. Next to this, small genomic aberrations (deletions, mutations) are commonly detectable in BCP-ALL. Among the most prominent examples are aberrations affecting the *IKZF1* gene. In approximately 15-20% of all pediatric BCP-ALL patients *IKZF1* aberrations can be detected, and several studies have shown that loss of *IKZF1* represents an independent poor prognostic factor in BCP-ALL. However, a direct link between loss of *IKZF1* and chemotherapy resistance had remained elusive at the start of this thesis work.

Since the start of my PhD project in 2014, much has been learned about the molecular mechanisms related to the effects of *IKZF1* loss on treatment outcome. **Chapter 2** of my thesis reviews the role of IKZF1 in chemotherapy resistance and summarizes recent findings on the potential molecular mechanisms underlying IKZF1-mediated chemotherapy resistance. In **chapter 3**, we established that single copy losses of *IKZF1* contribute to a poor response to synthetic glucocorticoids (GCs), both in genetic models as well as in patients. To examine whether loss of the tumor suppressor *IKZF1* directly causes resistance to chemotherapeutic agents commonly used in the treatment of BCP-ALL, we studied loss of *IKZF1* in a broad variety of model systems. We found that loss of *IKZF1* conferred a strong resistance to synthetic GCs both in B cells isolated from *Ikzf1*$^{+/-}$ mice and BCP-ALL cell lines displaying loss of IKZF1 function through knockout (CRISPR/CAS9) and knockdown (short hairpin RNA) approaches. Furthermore, analysis of historical *ex vivo* MTT data obtained from the Dutch Childhood Oncology Group revealed that *IKZF1*-deleted leukemic cells were significantly more resistant to GCs than wild type leukemic cells. Moreover, *IKZF1* deletions were highly enriched in leukemias showing a poor initial response to GC therapy (Prednisone day 8 response). Microarray gene expression analysis comparing wild-type versus *Ikzf1*$^{+/-}$ B cells with or without GC treatment revealed that the activation of GC-target genes is strongly diminished in *Ikzf1*$^{+/-}$ B cells. Notably, expression of *NR3C1* (glucocorticoid receptor, GR) was not altered in any of our model systems. These data suggest that resistance to prednisolone in *IKZF1*-deleted cells is not regulated at the level of *NR3C1* expression, but rather that mechanisms influencing GR activity or downstream signaling are responsible for the observed phenotype.

In **chapter 4** we describe how loss of IKZF1 reduces expression of protein phosphatase PTEN, leading to activation of its downstream effector AKT and AKT-specific phosphorylation of the GR at Ser134. The AKT inhibitor MK2206 significantly sensitized *IKZF1*-deleted cells to prednisolone. Moreover, primary patient samples carrying

aberrations in IKZF1 were also characterized by loss of PTEN protein expression and high phospho-GR levels. The GC resistance of these *IKZF1*-deleted samples could be reversed by treatment with the AKT inhibitor MK2206. Based on these data, we would like to further examine the effects of AKT inhibitor treatment on GC therapy response *in vivo*, using patient-derived xenograft models and explore whether high phospho-GR levels are a reliable biomarker for response to AKT inhibitors in GC-resistant, *IKZF1*-deleted ALL.

Despite the fact that *IKZF1* deletions are a poor prognostic marker in BCP-ALL, not all *IKZF1*-deleted leukemias respond poorly to therapy, suggesting that next to *IKZF1* deletions, also other co-occurring events may impact response to therapy. In **chapter 5**, we investigated which other gene deletions co-occurred in *IKZF1*-deleted BCP-ALL and whether these impacted the prognostic value of *IKZF1*. We demonstrated that single copy loss of tumor suppressor *BTG1*, as well as *CDKN2A*, *PAX5* and *RB1* deletions were significantly enriched in *IKZF1*-deleted BCP-ALL. Of these co-occuring lesions, only the combined loss of *IKZF1* and *BTG1* impacted the prognostic value of *IKZF1*. The combined presence of *IKZF1* and *BTG1* deletions appeared to be associated with a significantly lower event-free survival and higher cumulative incidence of relapse in pediatric BCP-ALL patients. To explore the cooperativity between copy number losses of *IKZF1* and *BTG1* in leukemogenesis, we examined the combined loss of *Ikzf1* and *Btg1* in a murine knockout model and observed that leukemogenesis in *Ikzf1*$^{+/-}$ mice was accelerated by the loss of *Btg1* in a *Btg1* dose-dependent manner. Moreover, combined loss of *Btg1* and 7 further enhanced GC resistance in murine splenocytes. These data provide compelling *in vivo* evidence that copy number losses in *Btg1* and *Ikzf1* cooperate during leukemia development and affect response to synthetic GCs.

In **Chapter 6** we explored the effects of *IKZF1* loss on response to different antimetabolites commonly used in the treatment of ALL and AML. We discovered that loss of IKZF1 in leukemic cell lines conferred resistance to pyrimidine analogs, synthetic nucleosides closely resembling the chemical structure of pyrimidines. Moreover, we found that *IKZF1*-deleted primary patient samples were highly resistant to pyrimidine analogs. We hypothesized that upregulation of cytidine deaminase (CDA) is responsible for pyrimidine resistance in BCP-ALL. Indeed, we established that CDA expression levels were increased in *IKZF1* knockout cells, accompanied by an increase in CDA activity in tumor cell lysates. Moreover, pharmacological inhibition of CDA restored pyrimidine analogue sensitivity in cell lines in which we reduced expression of *IKZF1* by shRNA mediated knockdown or CRISPR/CAS9 mediated deletion of *IKZF1*. Together these data point to a pivotal role for *IKZF1* copy number losses in mediating resistance to pyrimidine analogues.

Overall, the results in this thesis highlight an important role for IKZF1 in chemotherapy resistance. Further investigations should focus on approaches to effectively modify pathways acting downstream of *IKZF1* in order to enhance response to therapy.

General discussion

This thesis aimed to study the role of *IKZF1* aberrations in chemotherapy resistance in BCP-ALL by using *Ikzf1*-knockout mice, human ALL cell models lacking *IKZF1* as well as primary leukemia patient material. In addition, we investigated if and how *IKZF1* deletions cooperate with other co-occurring genetic events during leukemic transformation.

Loss of IKZF1 confers glucocorticoid resistance

GCs are crucial components in the treatment of ALL, and a poor response to GC therapy, is considered a predictor of poor outcome in BCP-ALL [1, 2]. GCs are highly effective drugs in the treatment of lymphoid malignancies and major efforts have been undertaken to improve their effect and to break therapy resistance.

One of the key findings in this thesis is the observation that loss of *IKZF1* contributes to glucocorticoid (GC) resistance, both in experimental models and in childhood leukemia patients [3] **(Chapter 3, 4 and 5)**. Other studies confirmed that *IKZF1* deletions are enriched in BCP-ALL leukemia patients showing GC resistance [4].

In our experiments aiming to model loss of *IKZF1* in leukemic cell lines, short hairpin RNA (shRNA)-mediated knockdown of *IKZF1* Exon 3 was effective in conferring GC resistance in BCP-ALL cell lines Nalm6, RS4;11 and ALL-PO **(Chapter 3)**. In contrast to this, Vitanza *et al* did not observe GC resistance in *IKZF1*-deleted leukemic BCP-ALL cell lines, which could be explained by the choice of intrinsically GC resistant cell lines (REH and SEM) in their study [3, 5]. Next to models reproducing loss of *IKZF1*, we observed that also primary *IKZF1*-deleted leukemic cells showed resistance to GCs *ex vivo*. Additionally, we found that *IKZF1*-deletions were significantly enriched in day 8 poor prednisone responders. However, not all patient samples were resistant to GCs *ex vivo*, which implies that other co-occurring genetic events could shape the resistance of *IKZF1*-deleted cells **(Chapter 5)**. For future studies, it is important to note that a broad variety of IKZF1 aberrations have been observed in BCP-ALL [6]. For example, it would be interesting to investigate the impact of the dominant–negative isoform *IK6* as well as of rare *IKZF1* deletions on GC therapy responses. Previously, loss-of-function but not dominant-negative *IKZF1* deletions have been associated with an adverse prognosis in adult acute lymphoblastic leukemia [7].

In our study, we analyzed historical data from ex vivo primary patient material treated with L-asparaginase, GCs, vincristine and 6-MP because those drugs are used during induction treatment [8]. However, these data historical data were limited to a restricted set of chemotherapies and these initial ex vivo drug screens did not contain combinations of therapies or novel therapeutic agents. Therefore, it would be interesting to screen more primary patient leukemia samples in the future using a larger panel of chemotherapeutic agents with state-of-the-art screening devices, in which a broad variety of novel

compounds, but also combinations of drugs, could be screened for their efficacy (Similar to our approach in **chapter 6**).

We further investigated in **Chapter 3** how loss of IKZF1 could lead to GC resistance and found that IKZF modulates the GC response by regulating Glucocorticoid Receptor (GR)-mediated gene expression. Still, how IKZF1 functionally regulates the GR remains elusive. A recent study has suggested that NR3C1 expression levels are reduced due to loss of IKZF1 [9], however in our studies **(Chapter 3 and 4)** we did not observe any changes in NR3C1 expression but rather diminished activation of GC-target genes, potentially due to impaired nuclear translocation of the GR after GC stimulation **(Chapter 4)**. Moreover, we observed direct regulation of GR-mediated transcription by IKZF1 on glucocorticoid responsive elements (GREs) in an GC-responsive luciferase reporter assay **(Chapter 3)**. Chromatin immunoprecipitation and coimmunoprecipitation experiments should help improve our understanding of the complex interactions between IKZF1 and the GR.

Our results show that intrinsic mechanisms of resistance affect the cells response to GCs in *IKZF1*-deleted BCP-ALL. However, other studies found that loss of *IKZF1* increases leukemic cell adhesion to the stromal niche [10], which has led to the suggestion that this adhesion phenotype may contribute to chemotherapy resistance in the bone marrow [11]. Recently, it was shown that IKZF1 regulates the expression of epithelial membrane protein 1 (EMP1), a protein implicated in GC resistance [12]. Knockdown of *EMP1* sensitizes BCP-ALL cells to prednisone while abrogating adhesion of leukemic cells to mesenchymal stroma cells (MSCs) [13]. The bone marrow microenvironment may alter chemotherapy responses of leukemic cells by secretion of pro-survival chemokines by MSCs [14, 15]. Therefore, leukemic cells showing a loss of *IKZF1* may interact with the bone marrow microenvironment to alter chemotherapy responses. The *in vitro* assays used in **chapter 3** did not include an MSC feeding layer that allowed crosstalk between MSCs and leukemic cells. Still, even in the absence of these microenvironmental influences, we observed GC resistance in *IKZF1*-deleted patient samples and model systems, arguing that microenvironmental influences may contribute to therapy resistance in *IKZF1*-deleted BCP-ALL, but that leukemia cell intrinsic mechanisms are most likely primarily responsible for the observed GC resistance phenotype.

An unexpected role for PTEN in *IKZF1*-deleted ALL

The PKB/AKT pathway is frequently deregulated in both solid and hematopoietic tumors [16]. Consistent with previous findings that suggests a role for AKT in GC resistance [17], we demonstrated in **Chapter 4** that AKT is activated in *IKZF1*-deleted leukemic cells, which is in agreement with studies of Chan et al [9]. We further observed that activation of AKT in *IKZF1*-deleted cells involves repression of the lipid phosphatase and tumor suppressor PTEN, which is a key regulator of AKT [18]. Transcriptional repression of *PTEN* by loss of IKZF1 function, appears to involve, at least in part, upregulation of HES1, a known repressor of PTEN function in T-ALL [19] (Figure 1). However, **Chapter 4** describes

that PTEN protein levels appear to be more strongly affected than PTEN mRNA levels, implying that PTEN expression is also regulated at a post transcriptional level. The notion that mutations in PTEN [20, 21], and its upstream regulatory pathway NOTCH [22-24], are almost exclusively found in T-ALL [25], suggests therefore that PTEN is rather influenced at the transcriptional and/or protein level in BCP-ALL. Our finding in the model systems we used point to potential transcriptional regulation of PTEN via HES1 in BCP-ALL similar to what is described in T-ALL [26], but interaction studies will be necessary to shed more light on this observation and to further understand the parallels between T-ALL and BCP-ALL (Figure 1).

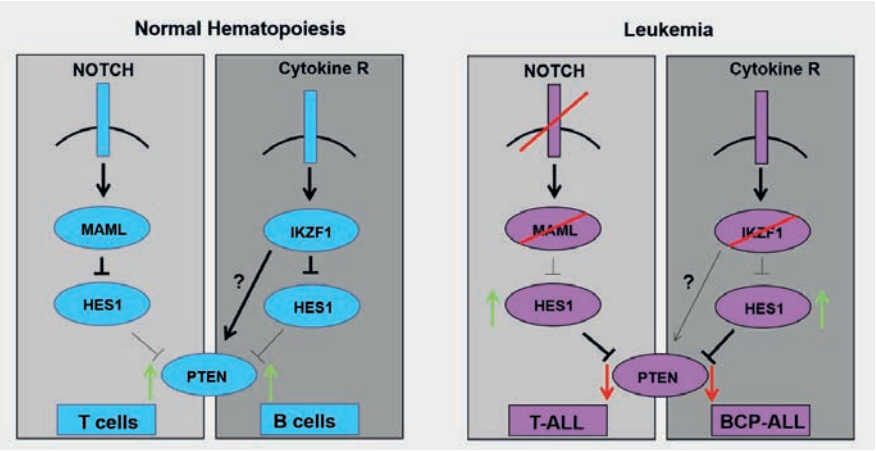


Figure 1 Schematic overview of the proposed parallels in the regulation of PTEN during normal hematopoiesis and leukemogenesis between T and B cell leukemia.

We observed that activation of AKT leads to increased levels of GR-Ser134 phosphorylation, which is linked to nuclear translocation of GR, in all of our *IKZF1* model systems as well as in patient-derived xenografts (PDX) **(Chapter 4)**. In line with our results, high GR-Ser134 phosphorylation has been correlated with GC resistance in T-ALL [17]. This further supports the notion that IKZF1 affects the GR-mediated gene expression rather on the activation level than on the expression level of the GR. Furthermore, analysis of phospho-GR protein expression levels in a larger cohort of BCP-ALL patients may provide more insight into the value of GR-Ser134 phosphorylation as potential biomarker for responsiveness to GC therapy in BCP-ALL.

Other studies have shown that inhibition of casein kinase II (CK2) enhances the transcriptional repressor function of IKZF1 [27,28]. As CK2 inhibits PTEN activation [29-31], it would be potentially interesting to investigate the role of CK2 in *IKZF1*-mediated loss of PTEN and how this impacts downstream AKT signaling.

We reported in **Chapter 4** that pharmacological inhibition of AKT via MK2206 successfully restored GC sensitivity both in *IKZF1*-deleted BCP-ALL cell lines as well as in primary leukemic cells. Despite the fact that clinical trials with MK2206 were discontinued due to insufficient clinical anti-leukemia activity when given alone at tolerated doses [32], monotherapies with novel AKT inhibitors as well as combination therapy of AKT inhibitors with additional therapeutic agents are under clinical evaluation [33]. As low dosages of MK2206 were sufficient to sensitize *IKZF1*-deleted cells to GCs in our studies, while not affecting overall cell viability, inhibition of AKT may represent a novel way to specifically enhance response to therapy in GC resistant *IKZF1*-deleted BCP-ALL cells. *In vivo* treatment of mice xenotransplanted with *IKZF1*-deleted leukemic cells may provide more insight into the potential beneficial effect of combined treatment with AKT inhibitors and GCs.

A role for BMI-1 in *IKZF1*-mediated GC resistance?

In addition to the regulation of the PTEN/AKT route via HES1 in *IKZF1*-deleted BCP-ALL, IKZF1 may also exert control over the PTEN/AKT signaling cascade by regulation of the B-lymphoma Mo-MLV insertion region 1 homolog gene BMI-1.

BMI-1 is a member of the Polycomb group (PcG) protein family and is critically involved in maintaining self-renewal of normal and malignant human stem cells [34]. Over the past years, BMI-1 has attracted interest as an oncogene; high expression of BMI-1 is correlated with a broad variety malignancies, including solid tumors [35-38] and hematopoietic malignancies [39-41]. Moreover, high levels of BMI-1 predict poor prognosis in BCP-ALL [42] and diffuse large B-cell lymphomas [43].

Preliminary data of our lab revealed that *IKZF1*^{KO} Nalm6 cells showed increased expression of BMI-1 after prednisolone treatment (Figure 2A). Next to this, treatment of IKZF1^{KO} Nalm6 cells with the BMI-1 inhibitor PTC209 [44] reversed GC resistance in those cells (Figure 2 B, C). Previous studies have already linked BMI-1 overexpression to drug resistance in B-cell lymphomas [45] and osteosarcoma [46]. Moreover, AKT-mediated phosphorylation of BMI-1 has been reported to alter its oncogenic function [47]. In **Chapter 4**, we report that the transcription factor HES1 protein expression is upregulated in IKZF1^{KO} cells. Interestingly, HES1 is known to activate BMI-1 [48] and to repress expression of PTEN [49,50]. Based on our finding that loss of IKZF1 leads to upregulation of HES1, we hypothesize that IKZF1 mediated regulation of PTEN involves repression by HES1. Because BMI-1 is functionally linked to both HES1 and PTEN [48,50], we speculate that BMI-1 could somehow be involved in this regulation. Future efforts should therefore aim to unravel the connection between BMI-1, HES1 and PTEN in *IKZF1*-deleted BCP-ALL.

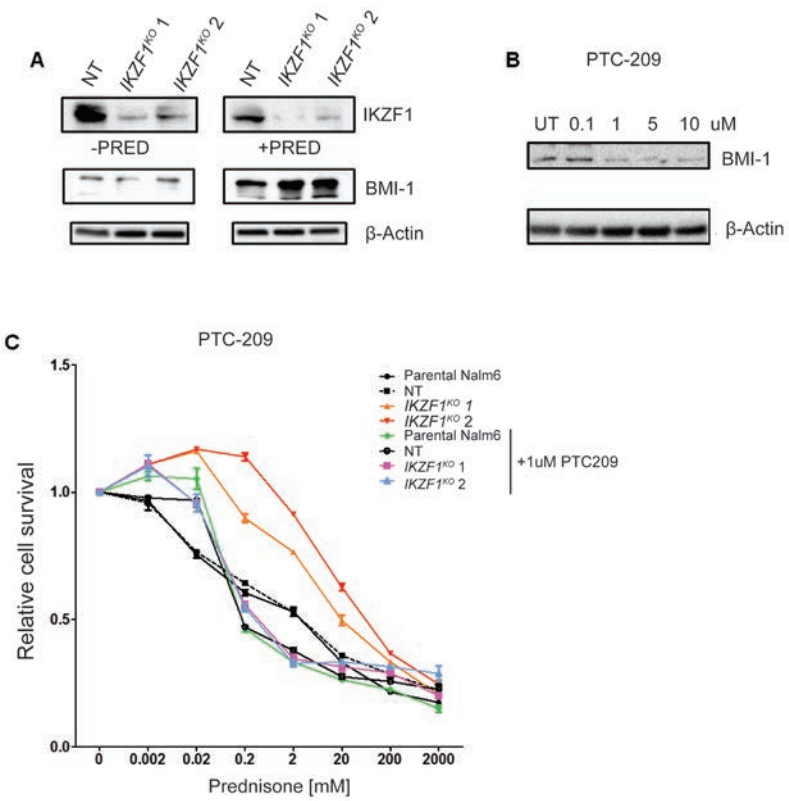


Figure 2 IKZF1^{KO} cells overexpress BMI-1 upon glucocorticoid treatment and are sensitive to BMI-1 inhibition (A) IKZF1 and BMI-1 protein expression levels of Nalm6 IKZF1 KO cell lysates after incubation with or without 2 μM Prednisone for 48 hours were analyzed by western blot. As controls, non-targeting Nalm6 cells were used while actin was used to control for loading. **(B)** BMI-1 protein levels after incubation with increasing concentrations of PTC209 for 48 hours analyzed by western blot. Actin was used as a loading control **(C)** Nalm6 parental, non-targeting controls or *IKZF1^{KO}* cells were treated for 48 hours with increasing concentrations of prednisolone with or without 1uM PTC209 and analyzed using an MTT based viability assay. All values were normalized to untreated Nalm6 cells. Error bars represent ± standard error of the mean (SEM).

Co-occurring genetic events in *IKZF1*-deleted BCP-ALL

Deletions affecting B-cell developmental genes such as *E2A*, *PAX5* and *IKZF1* are common events during lymphoid leukemogenesis and contribute to the development of leukemia [20,51]. *IKZF1* aberrations are among the most prominent reported alterations implicated in leukemia development. However, loss of *IKZF1* alone does not seem to be sufficient to induce leukemogenesis [52,53] and despite the fact that *IKZF1* deletions are enriched in relapsed ALL, the majority of patients with *IKZF1*-deleted BCP-ALL will not undergo relapse [54,55]. This indicates that next to *IKZF1* deletions, additional co-occurring genetic events may alter the impact of *IKZF1* alterations in BCP-ALL.

In **chapter 5**, we observed that single copy losses affecting tumor suppressor *BTG1* are enriched in patients with *IKZF1* deletion. Whereas deletions in *BTG1* alone did not have a prognostic value in our study cohort, combined loss of *BTG1* and *IKZF1* correlated with lower-event free survival and a higher incidence of relapse. Other common copy number losses such as *CDKN2A/B*, *PAX5*, *EBF1* and *RB1* were enriched in *IKZF1*-deleted BCP-ALL, but did not alter the outcome.

Both deletion of *IKZF1* and *BTG1* were shown to be a result of illegitimate RAG-mediated recombination [56] and activation- induced deaminase (AID) activity [57], which could explain that these lesions are prominent in specific BCP-ALL subgroups. Despite the fact that *BTG1* deletions are frequent event in BCP-ALL [56], there has been limited evidence showing that deletions affecting *BTG1* impact outcome. Only recently, *BTG1* deletions were found to be associated with a higher relapse risk in a large relapsed B-cell precursor ALL patient cohort treated on the international ALLR3 trial [58]. Furthermore, recent studies of the German BMF consortium support our initial findings that combined loss of *IKZF1* together with other genetic events can alter disease outcome of BCP-ALL [59]. However, in contrast to our findings, loss of *BTG1* had no significant influence on outcome in *IKZF1*-deleted leukemia in their so called IKZF1^{plus} high risk group. It will be important to compare several international study cohorts and also shed more light on effects of different national treatment protocols and population genetics to understand these cohort-dependent differences in more detail.

In **chapter 5**, we provided additional evidence for the tumor suppressive function of *Btg1* and *Ikzf1* in a genetic mouse model. *Ikzf1*$^{+/-}$ mice alone showed only a low incidence of lymphoid tumors during the monitoring period of 18 months. In contrast, transgenic *Ikzf1* mouse models expressing dominant negative isoforms of *Ikzf1* are highly susceptible for T-cell malignancies [60-62]. However, we observed that leukemia development was strongly increased by the combined loss *Btg1* and *Ikzf1* in a Btg1-dose dependent manner, indicating that the tumor suppressor genes *Btg1* and *Ikzf1* cooperate in leukemia development. Modeling the loss of B-cell specific tumor suppressor genes in relevant clinical models remains a challenge. Despite the fact that murine knockout models led to novel insights into the role of tumor suppressor genes in hematopoiesis and leukemogenesis, these mouse models do not always resemble the human phenotype. It is a commonly observed phenomenon of murine knockout mouse models for B-cell specific tumor suppressor genes that these mice develop T cell malignancies rather than expected B cell leukemias [63,64]. In **Chapter 5**, we also observed that loss of the two B-cell genes *Btg1* and *Ikzf1* in murine models exclusively led to T cell leukemias.

Loss of BTG1 enhances IKZF1-mediated GC resistance

Combined loss of *Btg1* and *Ikzf1* also enhanced the resistance of murine B-cells towards GCs, even surpassing the GC-resistance observed in *Ikzf1*$^{+/-}$ B cells. The underlying molecular mechanism remains elusive, as initial RNA sequencing experiments did not

reveal common pathways altered in *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ cells. However, in earlier work, our laboratory has identified BTG1 as a determinant of GC resistance in BCP-ALL [65]. It was shown to function as a transcriptional coregulator, as it has no catalytic activity. Instead, BTG1 regulates and interacts with the protein arginine methyltransferase PRMT1 [66,67]. We established that PRMT1 is able to associate with the promoter regions of both BTG1 and the GR in a BTG1-dependent manner, thereby influencing GR-mediated gene expression [65]. Preliminary experiments in our laboratory suggest that BTG1 and IKZF1 may be part of a complex (data not shown). Based on these observations, we postulate that BTG1 and IKZF1 interact either directly or via PRMT1 to fulfill their function, but further interaction studies will be needed to confirm this theory.

Loss of IKZF1 confers pyrimidine analog resistance

In **chapter 6** of this thesis, we observed that, apart from resistance to GCs, loss of *IKZF1* confers resistance to pyrimidine analogs. Limited numbers of xenografted patient material restricted the sample size of this study, and future efforts should focus on validating the observed resistance phenotype of *IKZF1*-deleted leukemic cells to pyrimidine analogs in a larger patient cohort. Furthermore, the current ALL-11 treatment protocol states treatment with Ara-C in four interval blocks of each 4 days between Day 34 and Day 62 of the treatment [68]. It would be interesting to study whether patients with a *IKZF1*-deleted leukemia depict a poor respond towards AraC during this interval in comparison to patients with wild-type *IKZF1* leukemias.

Although *IKZF1* is primarily a regulator of lymphoid development, there is some evidence supporting a role for this transcriptional regulator in myeloid differentiation: For instance, *IKZF1* has been implicated in megakaryopoiesis, which involves cooperation with transcriptional regulators such as *RUNX1* and *GATA1* [69]. Moreover, deletions in *IKZF1* were found to occur during progression from myeloid proliferative neoplasms to AML [70]. More recently, de Rooij et al showed that *IKZF1* deletions and point mutations are present in pediatric AML, while *IKZF1*-deleted cases consistently showed upregulation of GATA1 target genes [71]. CDA, the enzyme responsible for inactivation of AraC, is such an GATA1 target gene. Mutations leading to GATA1 loss of function are frequently found in down syndrome children with acute megakaryocytic leukemia (AMkL) and associated with strongly increased sensitivity to AraC [72]. This appears to be a direct consequence of the lack of *CDA* expression in these leukemias. Since CDA is subject to regulation by *GATA1* and IKZF1 is a repressor of GATA1 function, single copy loss of *IKZF1* in AML, by similarity to ALL, could lead to upregulation of *CDA* expression and consequently a poor response to AraC therapy (Figure 3). Indeed, deletion of chromosome 7, which harbors the *IKZF1* gene, is associated with poor outcome in AML [73], although this particular chromosome harbors other important leukemia genes, such as *EZH2*, that could affect outcome. We hypothesize, based on these findings and our results describe in **chapter 6**, that loss of *IKZF1*, both in ALL and AML, may contribute to AraC resistance by derepression of GATA1 activity (Figure 3). Given the key role of AraC in the treatment

of AML, future efforts should explore the relation between IKZF1, GATA1 and AraC resistance both in ALL and AML.

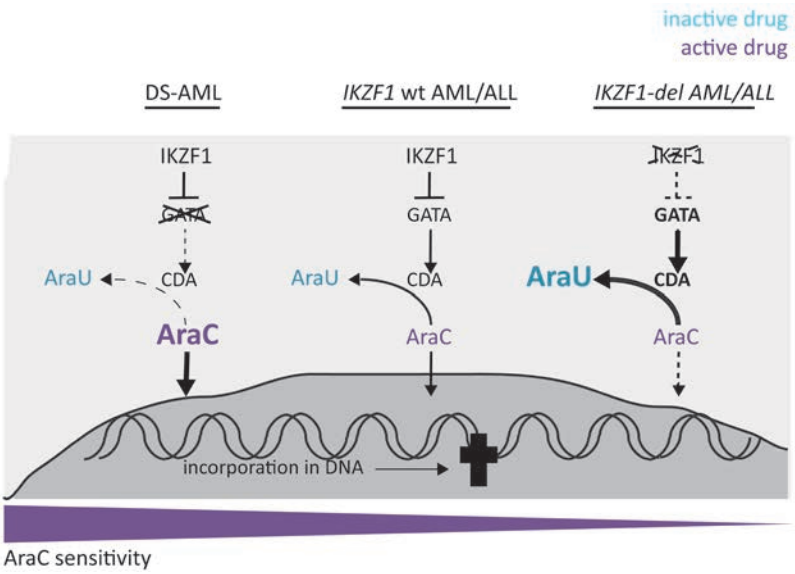


Figure 3 IKZF1 mediated control of CDA expression. Schematic overview of the proposed molecular mechanism that controls CDA expression

Overall Conclusion

This thesis provides novel insights into the contribution of lymphoid transcription factor IKZF1 to chemotherapy resistance in BCP-ALL. We show that loss of *IKZF1* confers resistance to synthetic GCs. Subsequently, we provide evidence that GC resistance in *IKZF1*-deleted model systems involves alterations in the PTEN/AKT signaling cascade. Moreover, we show that loss of *IKZF1* contributes to resistance to pyrimidine analogs. Furthermore, we observed that combined loss of *Ikzf1* and *Btg1* enhances murine leukemogenesis in a Btg1-dose dependent manner and that combined loss of *Ikzf1* and *Btg1* enhances GC resistance. We also show that BCP-ALL patients that show a combined loss of *IKZF1* and *BTG1* represent a novel risk group with a very poor survival outcome. From this thesis, it becomes evident that we need to specifically identify and target *IKZF1*-deleted tumors and that distinct mechanisms downstream of IKZF1 can affect the response to chemotherapy in *IKZF1*-deleted BCP-ALL. To decipher the most important pathways, screens with gRNA/CRISPR libraries as well as combinations of RNA-seq and proteomic approaches will be necessary to fully shed light on the impact of IKZF1 on (chemo)therapy resistance. Instead of targeting only one pathway, combining drugs hold promising potential to target minor chemoresistant subpopulations. Finally, this thesis shows that the genetic context in which *IKZF1* deletions occur is important and that next to the *IKZF1* status, also other co-occurring genetic events should be included to predict disease outcome.

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Chapter 8

Nederlandse samenvatting

Dankwoorden

Curriculum Vitae

List of Publications and Awards

PhD Portfolio

Nederlandse samenvatting

Voorloper B-cel leukemie, afgekort BCP-ALL, is de meest voorkomende vorm van kanker bij kinderen. In Nederland wordt deze diagnose gemiddeld bij 120 kinderen per jaar gesteld. In de laatste jaren hebben verbeteringen in de risico stratificatie en behandelingsprotocollen geleid tot een overlevingskans van 85-90% voor kinderen met BCP-ALL. Ondanks deze veelbelovende ontwikkelingen, ontstaat er bij 20% van alle patiënten met BCP-ALL een recidief. Dit kan direct gebeuren omdat de leukemie resistent is geworden tegen de behandeling, maar ook jaren na het bereiken van de remissie kunnen recidieven ontstaan omdat een dormante kleine populatie van leukemische cellen op later termijn opeens weer begint uit te groien. Om te begrijpen hoe dit recidief ontstaat, is het van belang te bergrijpen dat een tumor genetisch heterogeen is en meerdere en verschillende genetische afwijkingen bevat. Sommige van deze veranderingen in de genetische informatie van de tumor beinvloeden de overlevingskans van BCP-ALL patiënten en kunnen een voorspellende waarde hebben over het ziekteverloop. Eén van de voorbeelden van zo'n prognostische marker betreft genetische veranderingen (deleties of mutaties) in het *IKZF1* gen. Veranderingen in dit gen komen in 15-20 % van alle pediatrische BCP-ALL patiënten voor en meerdere studies hebben laten zien dat verlies van IKZF1 functie een onafhankelijke marker is voor een slechte prognose bij BCP-ALL. Alhoewel voorafgaand aan mijn promotieonderzoek IKZF1 genveranderingen als prognostische factor waren geïdentificeerd in BCP-ALL, was een directe link tussen verlies van IKZF1 functie en (chemo)therapie resistentie nauwelijks onderzocht. Mijn promotieonderzoek richt zich op deze resistentie tegen synthetische glucocorticoïden (GC), omdat GC behandelig een belangrijke deel is van de BCP-ALL behandeling. Resistentie tegen GCs is gecorreleerd met een slechte uitkomst van de behandeling. De mechanismen verantwoordelijk voor het optreden van GC resistentie zijn nog steeds grotendeels onbekend.

Hoofdstuk 1 omvat een algemene inleiding over de biologie van kinder ALL en hierin wordt de vraagstelling van mijn promotieonderzoek uiteen gezet. In **hoofdstuk 2** wordt een overzicht gegeven over de rol van IKZF1 op de ontwikkeling van leukemie en therapie resistentie, waarbij de mogelijke moleculaire mechanismen van IKZF1-gemedieerde therapie resistentie worden besproken. In de studie beschreven in **hoofdstuk 3** laten wij zien dat verlies van *IKZF1* functie bijdraagt aan een slechte respons tegen synthetische glucocorticoïden, zowel in genetische modellen in het laboratorium als ook in patiëntenstudies. Om te kunnen bepalen of genveranderingen van de tumorsuppressor *IKZF1* leiden tot therapie resistentie, hebben wij het verlies van IKZF1 in verschillende modelsystemen nagebootst. Wij vonden dat verlies van *IKZF1* leidt tot reistentie tegen de synthetische glucocorticoïden prednison en dexamethason, zowel in B cellen afkomstig uit *Ikzf1* knockout muizen als in BCP-ALL cellijnen De analyse van laboratorium en klinische data verkregen van de Stichting Kinderoncologie Nederland (SKION) laat zien dat leukemie cellen van patiënten met verlies van IKZF1 significant ongevoeliger zijn tegen corticosteroïden en dat IKZF1 deleties sterk verrijkt zijn in BCP-ALL patiënten met een slechte initiële response tegen therapie met glucocorticoïden. Met behulp van

genexpressie studies hebben we aangetoond dat in B cellen van *Ikzf1* knockout muizen genregulatie door glucocorticoïden is onderdrukt, terwijl expressie van de glucocorticoïd receptor zelf niet veranderd is.

Deze data suggereren dat glucocorticoïd resistentie in cellen met IKZF1 genveranderingen een gevolg is van verschillen in functionele activiteit van de glucocorticoïd receptor. Er zijn een aantal mechanismen beschreven die de effectiviteit van glucocorticoïd behandeling kunnen beïnvloeden. In T cel leukemie is verhoogde activiteit van de AKT signalerings route gerelateerd aan glucocorticoïd resistentie. Op basis van deze observaties hebben wij in de studies beschreven in **hoofdstuk 4** onderzocht of een verandering in AKT signalering verantwoordelijk is voor de glucocorticoïd resistentie van leukemie cellen met een *IKZF1* deletie. Wij hebben ontdekt dat verlies van *IKZF1* leidt tot gereduceerde expressie niveaus van het eiwit phosphatase PTEN. Dit resulteert vervolgens in activering van het kinase AKT en AKT-afhankelijke fosforylering van de glucocorticoïd receptor. Hierdoor is, na binding van glucocorticoïden, deze minder goed in staat om zich vanuit het cytoplasma naar de kern te verplaatsen. Het blijkt dat de AKT remmer MK2206 in staat is glucocorticoïd resistentie in *IKZF1*-gedeleteerde cellen op te heffen. Verder zien wij dat leukemiecellen van patiënten met verlies van IKZF1 gekenmerkt worden door lage eiwit expressie van PTEN and hoge phospho-glucocorticoid receptor niveaus. De glucocorticoïd resistentie in de leukemiecellen van deze patiënten kon in een aantal gevallen opgeheven worden door behandeling met MK2206. Op basis van deze resultaten zal nu het effect van AKT remming op glucocorticoïd resistentie in een muis model verder kunnen worden onderzocht. Ook kan worden onderzocht of hoge phospho-glucocorticoïd receptor expressie een betrouwbare biomarker is voor de effectiviteit van AKT remmers voor de behandeling van glucocorticoïd resistentie in patiënten met een *IKZF1* deletie.

Ondanks het feit dat *IKZF1* deletie een genetische marker is die geassocieerd is met een slechte respons op de behandeling, blijkt dat lang niet iedere leukemiepatiënt met een *IKZF1* deletie slecht reageert op therapie. Dit suggereert dat naast *IKZF1* deleties ook andere genetische veranderingen de prognose van *IKZF1*-gedeleteerde patiënten kunnen beïnvloeden. In het onderzoek beschreven in **hoofdstuk 5** tonen wij aan dat verlies van het BTG1 gen, maar ook deleties in *CDKN2A*, *PAX5* en *RB1*, significant verrijkt zijn in BCP-ALL met *IKZF1* deleties. Daarop hebben wij onderzocht of de prognostische waarde van *IKZF1* verlies wordt beïnvloed door combinaties met andere gen deleties. Verrassend genoeg vonden wij dat alleen het gecombineerde verlies van *BTG1* en *IKZF1* geassocieerd was met een lagere overleving en een hogere kans op een recidief in pediatrische BCP-ALL patiënten. Om verder de coöperatie tussen verlies van *BTG1* en *IKZF1* tijdens het ontstaan van leukemie beter te begrijpen, hebben wij het gecombineerde verlies van *Ikzf1* en *Btg1* in een muizen knockout model verder onderzocht. Wij vonden dat de leukemie ontwikkeling in heterozygote *Ikzf1* knockout muizen significant wordt versneld door verlies van Btg1 functie op een dosis afhankelijke manier. Daarnaast konden wij een sterkere glucocorticoïd resistentie in B cellen aantonen bij een gecombineerd verlies van *Btg1* en *Ikzf1* ten opzichte van *Ikzf1* verlies alleen. Samengevat betekenen deze resultaten

dat effecten van het verlies van *Btg1* and *Ikzf1* elkaar versterken tijdens de ontwikkeling van leukemie en dat deze samen de respons op glucocorticoïden onderdrukken.

Naast resistentie tegen glucocorticoïden zou IKZF1 ook de cellulaire respons op andere (chemo)therapeutica kunnen beïnvloeden. IKZF1 is recent ook beschreven als een 'metabole poortwachter' van de leukemische B cel. Op basis van deze observaties hebben wij in **hoofdstuk 6** het effect van *IKZF1* verlies op de gevoeligheid van verschillende antimetabolieten onderzocht. Onze data laten zien dat verlies van *IKZF1* in leukemische cellen leidt tot resistentie tegen pyrimidine analogen, synthetische moleculen die lijken op de bouwstenen van het DNA en de celdeling verstoren. Verder hebben wij ontdekt dat ook leukemie cellen verkregen uit patienten met een *IKZF1* deletie veel minder gevoelig zijn voor behandeling met het middel cytarabine, een pyrimidine analoog dat gebruikt wordt in de behandeling van patienten met ALL. Ook hebben wij aangetoond dat deze verminderde gevoeligheid voor cytarabine verband houdt met verhoogde expressie/ activiteti van het enzym cytidine deaminase (CDA). Dit enzym zorgt er namelijk voor dat chemotherapeutische metaboliten zoals cytarabine, worden omgezet naar een vorm die niet langer toxisch is voor de leukemiecel. Het blijkt inderdaad het geval dat zowel de activiteit als de mRNA expressieniveaus van CDA verhoogd zijn in *IKZF1* knockout cellen. Verder herstelde de farmacologische remming van CDA de gevoeligheid van BCP-ALL cellen voor cytarabine. Concluderend, wijzen deze resultaten erop dat er een belangrijke rol is voor *IKZF1* verlies in de relatie tot resistentie tegen pyrimidine analogen, als cytarabine, in BCP-ALL patiënten met een *IKZF1*-deletie. In **hoofdstuk 7** worden de resultaten van dit proefschrift in de vorm van een samenvatting en discussie gepresenteerd in relatie tot de relevante literatuur.

Samenvattend beschrijft dit proefschrift dat er een belangrijke rol is voor IKZF1 in chemotherapie resistentie bij BCP-ALL patiënten. Toekomstig onderzoek zou zich moeten richten op signalering routes die aangestuurd worden door IKZF1, zodat selectief de gevoeligheid voor therapie in patienten met *IKZF1*-gedeleteerde BCP-ALL verbeterd kan worden.

Dankwoord

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Curriculum Vitae

René Marke was born on the 5th of November 1987 in Mönchengladbach, Germany. After finishing high school at the Gymnasium Korschenbroich and his civilian service as a paramedic in Germany, he started a study of biology in the neighbouring country, the Netherlands, at the Radboud University Nijmegen in 2008. During this time, he focussed on medical biology and performed his Bachelor internship at the department of biochemistry at the Radboud Institute for Molecular Life Sciences (RIMLS) under supervision of Dr. Merel Adjobo-Hermanns and Prof. Dr. Roland Brock. Afterwards, he entered the selective Master's programme: "Molecular mechanisms of diseases" at the Radboud university medical center (Radboudumc) Nijmegen in 2011.

During this time, his passion for oncology research ignited. He performed his first Master Internship at the department of cell biology (under supervision of Dr. Katarina Wolf and Prof. Dr. Peter Friedl) at the RIMLS, in which he performed research on tumor cell migration and invasion. For his 2nd Master internship, he travelled to Toronto, Canada to study acute myeloid leukemia in the laboratory of Prof. Dr. John Dick. His work in Toronto contributed to two publications in Nature and one publication in Cancer Cell. After finishing his Masters in 2013, he moved back to the Netherlands and started his PhD at the department of pediatric oncology at the Radboudumc Nijmegen under supervision of Dr. Frank van Leeuwen, Dr. Blanca Scheijen and Prof. Dr. Peter Hoogerbrugge. The research revolved around chemotherapy resistance in acute lymphoblastic leukemia and has been described in this thesis.

During his PhD trajectory, he supervised five students and attended several national and international conferences. He was awarded a Young Investigator Award from the International Society for Pediatric Oncology (SIOP) to travel to Toronto to present his research in 2014. Together with other awardees, he launched a Young Investigator Network from and for young oncologists as well as scientists and was an active board member from 2014 to 2018. In the autumn of 2017, René received the Tom Voûte Young Investigator Award. This price is awarded each year by Kika (kinderen kankervrij) for excellent research in the area of pediatric oncology. After several years of fundamental research, René chose to perform a training as clinical research associate (CRA) in Germany which he completed with a score of 1.0. Since autumn 2018, he is part of the exciting world of clinical trials and works as a clinical project manager. He lives happily together with his wife Samira, their little dog Dobby and their newborn daughter Lotta in Mönchengladbach, Germany.

List of Publications

Thesis related

René Marke*, Jørn Havinga*, Jacqueline Cloos, Marc Demkes, Geert Poelmans, Laurensia Yuniati, Dorette van Ingen Schenau, Edwin Sonneveld, Esmé Waanders, Rob Pieters, Roland P. Kuiper, Peter M. Hoogerbrugge, Gertjan J.L. Kaspers, Frank N. van Leeuwen, Blanca Scheijen. *Tumor Suppressor IKZF1 Mediates Glucocorticoid Resistance in B cell Precursor Acute Lymphoblastic Leukemia.* **Leukemia. 2016 Jul; 30(7):1599-1603.** doi: 10.1038/leu.2015.359

Blanca Scheijen*, Judith M. Boer*, **René Marke***, Esther Tijchon, Dorette van Ingen Schenau, Esmé Waanders, Liesbeth van Emst, Laurens T. van der Meer, Rob Pieters, Gabriele Escherich, Martin A. Horstmann, Edwin Sonneveld, Nicola Venn, Rosemary Sutton, Luciano Dalla-Pozza, Roland P. Kuiper, Peter M. Hoogerbrugge, Monique L. den Boer, Frank N. van Leeuwen (2017) *Tumor suppressors BTG1 and IKZF1 cooperate during mouse leukemia development and increase relapse risk in B cell precursor acute lymphoblastic leukemia patients.* **Haematologica March 2017 102: 541-551**; doi:10.3324/haematol.2016.153023

René Marke, Frank N. van Leeuwen, Blanca Scheijen. **The many faces of IKZF1 in B cell precursor acute lymphoblastic leukemia. Haematologica. 2018 Apr;103(4):565-574.** doi: 10.3324/haematol.2017.185603

Miriam Butler*, **Rene Marke***, Dorette van Ingen Schenau, Jean Pierre Bourquin, Beat Bornhauser, Silvia Jenni, Blanca Scheijen, Laurens van der Meer*, Frank van Leeuwen*. *Loss of IKZF1 confers resistance to pyrimidine analogs in B cell precursor acute lymphoblastic leukemia*. **Manuscript in preparation**

René Marke*, Miriam Butler*, Dorette van Ingen-Schenau, Blanca Scheijen, Frank N van Leeuwen. *PTEN/AKT Pathway drives Glucocorticoid Resistance in IKZF1-deleted B Cell Precursor Acute Lymphoblastic Leukemia*. **Manuscript in preparation**

*These authors contributed equally to this work

Others

Liran I. Shlush, Sasan Zandi, Amanda Mitchell, Weihsu Claire Chen, Joseph M. Brandwein, Vikas Gupta, James A. Kennedy, Aaron D. Schimmer, Andre C. Schuh, Karen W. Yee, Jessica L. McLeod, Monica Doedens, Jessie J. F. Medeiros, **René Marke**, Hyeoung Joon Kim, Kwon Lee, John D. McPherson, Thomas J. Hudson, The HALT Pan-Leukemia Gene Panel Consortium, Andrew M. K. Brown, Fouad Yousif, Quang M. Trinh, Lincoln D. Stein, Mark D. Minden, Jean C. Y. Wang & John E. Dick (2014) *Identification of pre-leukaemic*

haematopoietic stemcells in acute leukaemia. **Nature. 2014 Feb 20;506(7488):328-33**. doi: 10.1038/nature13038

Eric R. Lechman, Bernhard Gentner, Peter van Galen, Stanley Wai-Kwong Ng, Kolja Eppert, **René Marke**, Liran Slush, James A. Kennedy, Amanda Mitchell, Weihsu Claire Chen, Veronique Voisin, Gary Bader, Mark D. Minden, Jun Lu, Jean C.Y. Wang, Luigi Naldini, John E. Dick. (2016) *Mir-126 Governs Human Leukemia Stem Cell Quiescence and Chemotherapy Resistance*. **Cancer Cell. 2016 Feb 8;29(2):214-28**. doi: 10.1016/j.ccell.2015.12.011.

L. Shlush *, Amanda Mitchell *, Lawrence Heisler, Sagi Abelson, Stanley W. K. Ng, Aaron Trotman-Grant, Jessie J. F. Medeiros, Abilasha Rao-Bhatia, Ivana Jaciw-Zurakowsky, **René Marke**, Jessica L. McLeod, Monica Doedens, Gary Bader, Veronique Voisin, ChangJiang Xu, John D. McPherson, Thomas J. Hudson, Jean C. Y. Wang, Mark D. Minden & John E. Dick (2017) *Tracing the origins of relapse in acute myeloid leukaemia to stem cells.* **Nature. 2017 Jul 6;547(7661):104-108**. doi: 10.1038/nature22993

*These authors contributed equally to this work

Awards

09/2017	Tom Voûte Young Investigator Award 2017
01/2016	RIMLS Award 2016: International Travel Grant
08/2015	Radboud University International Travel Grant 2015
10/2014	SIOP Young Investigator Award 2014

Institute for Molecular Life Sciences
Radboudumc

PHD PORTFOLIO

Name PhD candidate: ***René Marke***
Department: ***Laboratory of pediatric Oncology***
Graduate School: ***Radboud Institute for Molecular Life Sciences***

PhD period: ***01-01-2014 until 31-03-2018***
Promotor(s): ***Prof. P.M. Hoogerbrugge***
Co-promotor(s): ***Dr. F.N. van Leeuwen***
Dr. B. G.P.H. Scheijen

		Year(s)	ECTS
TRAINING ACTIVITIES			
a)	**Courses & Workshops**		
	Introduction day Radboudumc	2014	0.5
	Graduate School specific introductory course (RIMLS)	2014	2.0
	Scientific Integrity course	2015	2.0
b)	**Seminars & lectures**		
	Dr Brian Gloss: Trends in Transcriptomics	2014	0.1
	Prof. Stephen Bustin: Real-time quantitative PCR: toward greater transparency and reproducibility	2014	0.1
	Dr. Iñaki Martin-Subero: DNA methylation landscapes of normal and neoplastic hematopoietic cells	2014	0.1
	Prof. Matthew Bogyo: Using chemical tools to track and target proteases involved in cancer, inflammation and infection	2014	0.1
	CRISPR Cas genome editing: CRISPR Cas genome editing is a lecture of the Graduate Course..	2014	0.1
	Prof. Hidde Ploegh Imaging immunity: antibody fragments and their enzymatic modification	2015	0.1
	Dr. Francesca Cesari Science publishing - behind the scenes at Nature	2015	0.1
	Prof. René Medema Determinants of cell fate after DNA damage	2015	0.1
	Prof. Peter Hillmen Targeted Therapy in Haematology: A First Step to Cure	2015	0.1
	Dr. Jan-Michael Heinrich The pluriSelect technology: Separating multiple cell types in one step	2015	0.1
	Organizing Technical Forum (Grant Writing)	2016	0.5
c)	**Symposia & congresses**		
	2014		
	Radboud Frontiers	2014	1.0
	PhD retreat + Poster	2014	1.0
	Radboud Oncology day + Oral	2014	0.75
	National congresses		
	Dutch Hematology Congress 2014+ Oral	2014	1
	KWF Tumor Biology meeting 2014 + Oral	2014	0.75
	International congresses		
	International Society for Pediatric Oncology (SIOP) conference 2014, Toronto + Oral	2014	1.5
	2015		
	PhD retreat + Poster	2015	1.0
	Radboud Frontiers	2015	1.0
	National Congresses		
	Dutch Hematology Congress 2015+ Oral	2015	1.0
	KWF Tumor Biology meeting 2015 + Oral	2015	0.75

		Year(s)	ECTS
	International congresses		
	International Society for Pediatric Oncology (SIOP) conference 2015, Capetown + Oral	2015	1.5
	Co-organization of the Scientific day for Young Investigators during the SIOP 2015 in Capetown	2015	2.0
	2016		
	PhD retreat+Poster	2016	1.0
	Co-organization of the Scientific day for Young Investigators during the SIOP 2016 in Dublin	2016	2.0
	International congresses		
	ASH San Diego 2016	2016	1.0
	International Society for Pediatric Oncology (SIOP) conference 2016, Dublin + Oral	2016	1.5
	National congresses		
	KWF Tumor Cell biology meeting 2016	2016	0.5
	RIMLS New Frontiers	2016	1.0
	Rotterdam Molecular Hematology Meeting + Oral	2016	0.75
	2017		
	PhD retreat 2017 + Oral	2017	1.0
	Co-organization of the Scientific day for Young Investigators during the SIOP 2017 in Washington DC	2017	2.0
	National congresses		
	Rotterdam Molecular Hematology Meeting 2017+ Oral	2017	0.75
	KWF Tumor Cell biology meeting 2017 +Oral	2017	0.75
TEACHING ACTIVITIES			
d)	**Supervision of internships / other**		
	Supervising Master Student Marissa van Maaren	2014	2
	Supervising Master Student Deniz Pirincci	2015	2
	Supervising Honours Student Janneke Elzinga	2015	1
	Supervising Master Student Pascal Vos	2016	2
	Supervising Bachelor Student Denize van den Beemt	2017	2
TOTAL			**40.5 EC**

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